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Inflammation in Prostate Carcinogenesis: Role of the Tumor Suppressor Par-4

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Prostate cancer (PCa) is one of the most common malignancies in men, and is a complex disease in its development and response to therapy. Loss of tumor suppressors genes is a frequent initiating event that is irreversible, whereas tumor promotion and progression are susceptible to modulation, which provides a rationale for therapeutic intervention. Tumor promotion is highly regulated by the interaction between initiated cells and their microenviroment and inflammation is a frequent and important tumor promoter. However, despite the strong evidence for an inflammatory component to the pathology of PCa, the process of inflammation and the related signaling pathways are largely unknown. Therefore, a better understanding of the molecular mechanisms that govern the inflammatory response and its impact on PCa progression is of paramount importance in developing novel therapies for PCa. Here we report the identification of a novel network between tumor suppressors: Par-4, PKCζ and PTEN and the characterization of the inflammatory response unleashed upon their loss. Importantly, our results also demonstrate that inflammation is not only secondarily associated to carcinogenesis but it is an important contributing factor in tumor promotion.

15. SUBJECT TERMS

Tumor suppressors, PKCζ, Par-4, PTEN, NF-κB, inflammation, IL-6, Arg1, iNOS, prostate cancer

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Introduction

Prostate cancer (PCa) is one of the most common malignancies in men In developed countries, and it ranks third overall in terms of mortality (behind lung cancer and colon cancer) (1). PCa is a complex disease in its development and response to therapy (2). Therefore, the development of new therapies and better diagnostic techniques will depend on increasing our understanding of the molecular basis of this disease.

Prostate carcinogenesis is a multistage process that includes initiation, promotion and progression (3). Loss of tumor suppressors genes is a frequent initiating event that is irreversible, whereas tumor promotion and progression are susceptible to modulation, which provides a rationale for therapeutic intervention (4). Tumor promotion is highly regulated by the interaction between initiated cells and their microenvironment (5, 6). Furthermore, recent published studies have proposed that inflammation could be a frequent tumor promoter in many types of cancers, including PCa, although the mechanisms underlying these processes remained poorly characterized. Therefore, a better understanding of the molecular mechanisms that govern the inflammatory response and its impact on PCa progression is of paramount importance in developing novel therapies for PCa. The major goal of this project was to investigate how inflammation promotes tumor progression using a novel genetic mouse model that involves the loss of two tumor suppressors, Par-4 and PTEN (7-9). Two main questions were addressed: 1) how do Par4 and PTEN cooperate to promote PCa, and 2) how does inflammation promote tumor progression upon loss of these tumor suppressors.

Body

Please note that this award was transferred from the University of Cincinnati to the Sanford-Burnham Medical Research Institute (SBMRI). Due to the administrative process for this transfer, funds were not available during the period of March 2011-July 2011. Therefore, a no-cost extension of 2 months was approved to allow the completion of the project. A revised SOW was approved during the transfer, expanding tasks from those initially planned. As described bellow, we have successfully completed the whole project including the new tasks described in the revised SOW with great results and productivity (see outcomes).

Task 1: The breeding of WT, PTEN+/-, Par-4 KO, PTEN+/- Par-4+/- and PTEN+/- /Par-4 KO was already performed in the first and second years. We extended our study to the compound mutant PTEN+/-/PKCζ KO due to the difficulties in generating PTEN+/-/p53+/- mice. During this last period, we finally generated double mutant PTEN+/-/p53+/- mice, however, all these mice presented limited survival, dying of lymphadenopathies at young ages before developing prostate adenocarcinoma, which precluded further analysis to compare with the double mutant PTEN+/- Par-4 KO. Therefore, the project focused on the analysis of the double mutant lines PTEN+/- Par-4 KO and PTEN+/-/PKCζ KO.

Task 2: During the first and second year of this award, we fully completed Task 2 of the original SOW and Task 2a and partially Task2b of the revised SOW. This work led to the characterization of the prostate phenotype of PTEN/Par4 double mutant mice as a novel mouse model for prostate cancer progression ((10) see Appendix). Thus, we found that loss of Par-4 cooperated with PTEN heterozygosity to promote invasive carcinoma (10). The loss of both tumor suppressors was sufficient to promote invasive carcinoma, but not metastasis (2nd year report).

During the second year of the project, we also characterized the prostate phenotype of PTEN+/-/PKCζ KO double mutants. Interestingly, these results unveiled a role of PKCζ as a tumor suppressor in prostate cancer (2nd Year report).

The next step in our project was to test the hypothesis that inflammation is a key mediator of the increased prostate neoplasias that result from Par-4 deficiency. This was performed in Task 2 (revised SOW). Completed Task 2a demonstrated increased NF-κB activity in PTEN/Par4 double mutant prostates as compared to PTEN+/- prostates, measured as immunostaining (IHC) of RelA to determine activation of NF-κB by nuclear translocation (10). In addition, we extended this characterization to phospho-RelAS276 and phospho-IKKα/β IHC as subrogate well-established markers of NF-kB activation (10). Furthermore, Par-4 deficiency led to no changes in ERK activation, measured as pERK IHC, but to an increase in AKT activation in the prostates of the PTEN+/- /Par-4 KO double mutants. Thus, the concomitant loss of PTEN and Par-4 impinges both in the NF-κB cascade and in AKT(10).

We next sought to characterize the immune response in Par-4 deficient and PTEN+/- /Par-4 KO mutant prostates, as planned in Task 2b (Revised SOW). First, we determined NF-kB-dependent genes and inflammatory genes by Q-PCR on RNA derived from prostates of the different mutants. We found synergistic activation of IL-6, TNFα, Fhc and KC (murine homolog of IL-8) in the prostates of the double mutants, consistent with the enhanced activation of this pathway (10). Since IL-6 and IL-8 could promote angiogenic signals, we also detected an increased in angiogenesis in the double mutants as determined by CD31 IHC (1st year report-unpublished data). However, no significant differences were observed in mRNA levels of Vegf-A, Vegf-C, Vegf-R3 or Pecam-1 in PTEN+/-/Par-4 KO prostates as compared to those of PTEN+/- (2nd Year Report).

To evaluate the cellular immunobiology of the different mutant prostates, we initially determined the infiltration of immune cells by H&E staining. There was an increase in infiltrated immune cells in both PTEN+/- /Par-4 KO (1st Year report) and PTEN+/-/PKCζ KO (2nd Year Report) prostates as compared to PTEN+/-, suggesting a process of chronic inflammation in the prostates of the double mutants. Since these are total constitutive knock-outs, we investigated whether these tumor suppressors could cooperate in the immune response as a potential mechanism to impact prostate carcinogenesis. Analysis of the immune organs revealed spleen and lymph nodes enlargement, enhanced proliferation and altered splenic architecture in PTEN+/- /Par-4 KO mice (1st Year Report). These results suggest that a systemic immune response could impact the development of prostate carcinoma in these mice.

In fact, an adoptive transfer experiment generating chimeras of PTEN+/- reconstituted with the immune system of either PTEN+/- or PTEN+/-Par-4 KO mice resulted in higher incidence of invasive carcinoma in the mice reconstituted with the immune system of the double mutant, as compared to the ones with that of PTEN+/-. This key experiment points to a promoting effect of the immune system-driven inflammation in the induction of the invasive phenotype in the double mutant (2nd Year Report).

To better characterize the inflammatory response in the prostate, we performed IHC and Q-PCR analysis for the different immune cell populations. Thus, staining for the T and B cell markers CD3 and B220 in prostate sections of these mutants demonstrated a significant recruitment in T and B cells in both double mutants as compared to PTEN+/- (1st and 2nd Year Reports). However, no changes in recruitment of macrophages, as determined by Q-PCR analysis of the macrophage marker CD68, were detected in the double mutant prostates (**Fig. 1**). Furthermore, we determined a set of cytokine expression by Q-PCR. No changes were found in IL-12, whereas IFNγ was increased in the double mutants. Levels of IL-4, IL-17 and IL-23 were undetectable (1st Year report). Analysis of macrophage activation markers revealed that although there was not an increase in macrophage recruitment (Fig.2), however there was a general activation state of macrophages in the prostates of the double mutant. That is, there was a synergistic increase in iNOS (M1 activation marker) and Arg1 (M2 activation marker) mRNA levels (2nd Year Report).

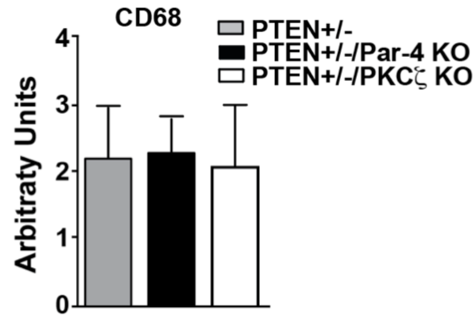


Fig. 1. Macrophage infiltration is not altered in PTEN+/-/Par-4 KO or PTEN+/-/PKCζ KO prostates.
Quantification of Q-PCR of mRNA levels of the macrophage marker CD68 in prostates from 6 month-old mice of the different genotypesResults are shown as mean ±SD. n=5 mice.

It has been reported that high levels of iNOS and Arg1 have suppressive action on tumor-infiltrating lymphocytes by increasing L-arginine metabolism to product peroxynitrites (11). This is consistent with our finding that there was an increase in T cell recruitment in the double mutant but this was not associated with a T cell activation response. Since it has been proposed that the increase in iNOS and Arg1 could be an anti-tumor response produced not only by the activation of the macrophages but also by the prostate cancer cell, we determined the source of these enzymes by IHC in prostate sections of the different mutants. Consistent with the Q-PCR data, we found an increase in iNOS and Arg1 staining in prostates of both double mutants as compared to those of PTEN+/- (**Fig. 2**). Interestingly, this staining is compatible with the tumor cells being the source of both enzymes.

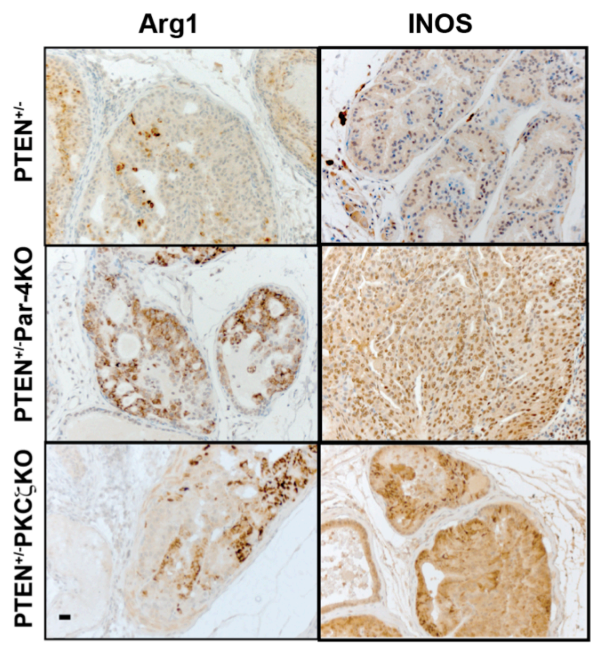
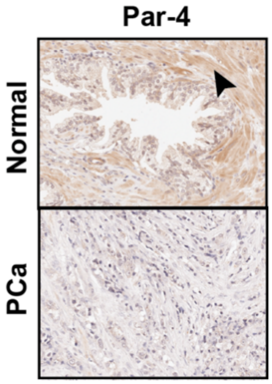


Fig. 2. Increased Arg1 and INOS in PTEN+/-/Par-4 KO and PTEN+/-/PKCζ KO prostates.
Immunostaining for Arginase 1 (Arg1) and nitric oxide synthase (iNOS) of prostates from 10-month-old mice of different genotypes. n=5 mice. Scale bar, 20 μm.

Task 3: Stromal cells are known to stimulate epithelial cell growth and contribute to the genesis and progression of tumorigenesis (12, 13). Therefore, we explored the role that the stroma plays in the elicited inflammatory response caused as a consequence of Par-4 loss. To do that, we first determined whether Par-4 is expressed in CAFs. As shown in **Fig. 3**, Par-4 is expressed in both αSMA and vimentin positive cells, suggesting a potential role in CAFs. Of note, Par-4 is also highly expressed in human



	Par-4 in stroma		
	Low *n* (%)	High *n* (%)	*p*-value Chi^2
Gleason Score (*n*=189)			
normal-4	31(20)	13(38)	*p*=0.022
5-10	124(80)	21(62)	

Fig. 4. Par-4 in stroma. Par-4 is highly expressed in normal and low Gleason score PCas, and is lost in high Gleason socre. Immunostaining of Par-4 in human prostate samples. A TMA containing 162 PCa samples and 27 matching normal tissue was analyzed for Par-4 expression in stroma. Arrow indicates strong staining in stroma.in normal tissue.

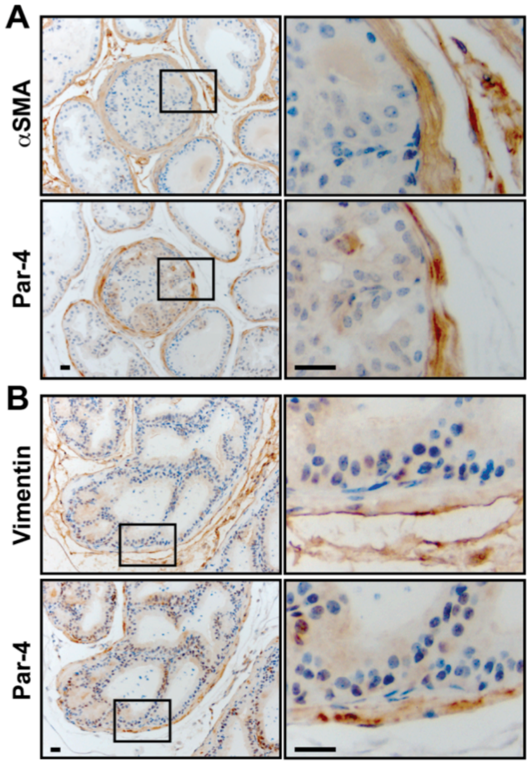


Fig. 3. Par-4 is expressed in stroma. Immunostaining for CAFs markers aSMA(A) and Vimentin (B) and Par-4, in consecutive sections of prostates from 4-month-old PTEN+/- mice. n=5 mice. Scale bar, 20 μm.

prostate (**Fig. 4**). Interestingly, staining of prostate sections of the different mutants with αSMA showed a marked decrease of αSMA expression in prostates of the double mutant, consistent with its invasive phenotype (**Fig. 5**). Of note, characterization of Par-4 expression in the stroma of human prostate samples revealed that Par-4 levels in the stroma were lost upon progression to high-grade Gleason score (**Fig. 4**). We then determined the expression of the following stromal genes: SDF-1 and its receptors CXCR4 and CXCR7; TGF-b and BMPS (BMP2, BMP4 and BMP7). Interestingly, we detected a significant increase in CXCR4 and TGF-b (**Fig. 6**). This finding will require further research beyond the goal of this project to unveil how Par-4 deficiency impinges in these pathways in the stroma. Finally, we tested whether the stroma could be the source of IL-6 in the double mutants. To test that, we performed IHC in consecutive sections of prostates from the double mutants. As shown in **Fig. 7**, IL-6 is mostly produced by tumor epithelial and inflammatory cells in the double mutant prostates, with no significant contribution of the stroma to the release of this cytokine. These results suggest that Par-4 deficiency in the stroma most probably will use the TGF-b pathway to impact carcinogenesis. Of note, it has been shown that TGF-b signaling in fibroblasts could modulate the oncogenic potential of adjacent epithelia (14).

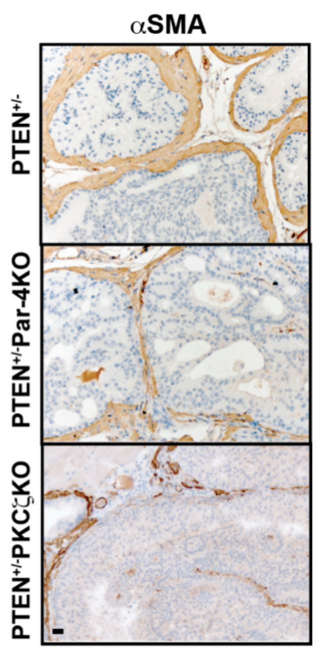


Fig. 5. Reduced αSMA expression in PTEN+/-/Par-4 KO and PTEN+/-/PKCζ KO prostates. Immunostaining for αSMA of prostates from 10-month-old mice of different genotypes. n=5 mice. Scale bar, 20 μm.

<u>Task 4:</u> The aim of this task was to validate the role of Par-4 and its associated inflammatory response in human prostate tumors. Our previous results (2nd Year Report) demonstrated that there was a significant association between the loss of both tumor suppressors, which is consistent with a cooperative function of Par-4 and PTEN in vivo in human PCa samples. In addition, our results demonstrated that there was a significant

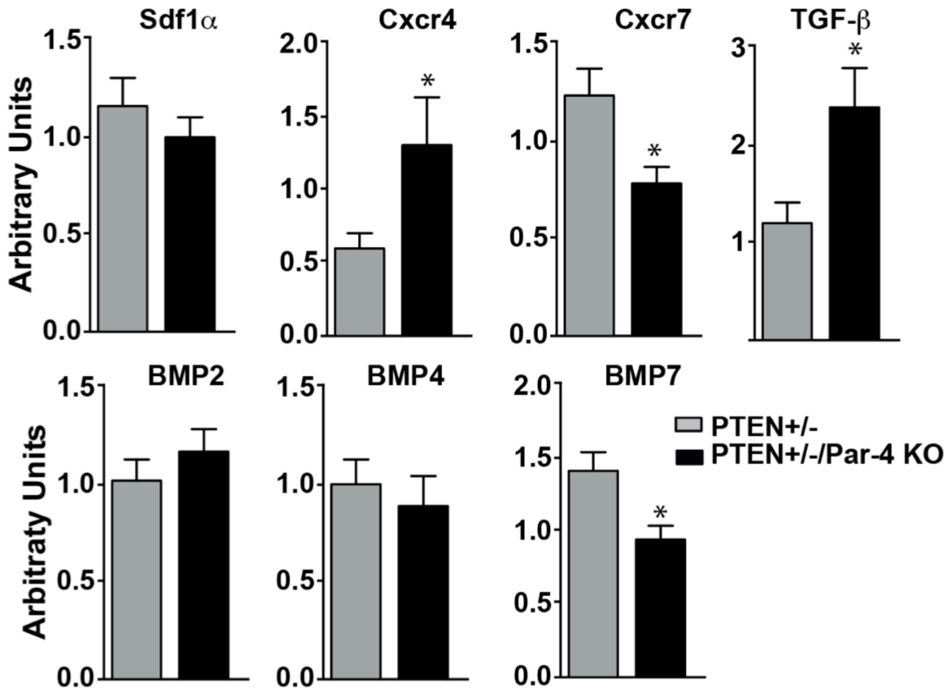


Fig. 6. mRNA levels of stromal genes in PTEN+/-/Par-4 KO prostates. Quantification of Q-PCR of mRNA levels of stromal genes in prostates from 6 month-old mice of the different genotypes. Results are shown as mean ±SD. n=5 mice. *, p< 0.05

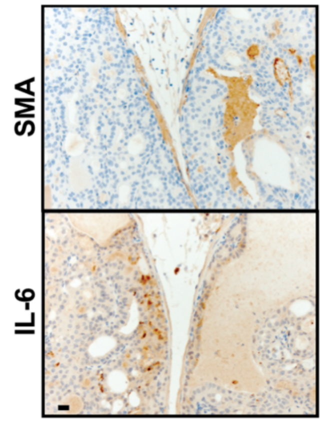


Fig. 7. IL-6 in PTEN+/-/Par-4 KO prostates. Immunostaining for SMA and IL-6 in consecutive sections of prostates from 10-month-old PTEN+/-/Par-4 KO mice. n=5 mice. Scale bar, 20 μm.

negative correlation of IL-6 levels with both Par-4 and PTEN expression (2nd Year Report). These results indicate that IL-6 could be a critical mediator of the mechanism of action of the cooperation of the tumor suppressors Par-4 and PTEN.

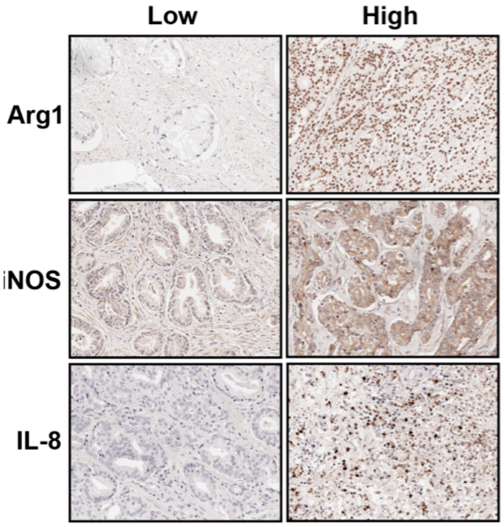
During this last period, we have extended our study to the analysis of Arg1, iNOS and IL-8, which were found to be significantly increased in the double mutants. We used three TMAs from US Biomax consisting of 256 adenocarcinoma and 35 normal prostate samples. Of note, analysis of correlation of the different stainings along with Gleason score revealed that whereas Par-4 is lost upon Gleason score progression, Arg1, iNOS and IL-8 all of them positively correlated with Gleason score (Tables, below). Interestingly, we have also found a significant correlation of Par-4 levels with Arg1 and iNOS expression, whereas no significant association was found with IL-8 (**Fig. 8)**. Therefore, these results suggest that inhibitors that counteract these two key enzymes of the L-Arginine metabolism could be helpful to activate T cell function and block prostate cancer progression.

	Par4		
	Low	High	p-value
	n (%)	*n* (%)	Chi^2
Gleason Score (*n*=256)			
2-7	39 (34.8)	69 (47.9)	*p*=0.0353
8-10	73 (65.2)	75 (52.1)	

	Arg1		
	Low	High	p-value
	n (%)	*n* (%)	Chi^2
Gleason Score (*n*=256)			
2-6	39 (28.7)	20 (16.7)	*p*=0.0228
7-10	97 (71.3)	100 (83.3)	

	iNOS		
	Low	High	p-value
	n (%)	*n* (%)	Chi^2
Gleason Score (*n*=256)			
2-6	3 (60.0)	56 (22.3)	*p*=0.0475
7-10	2 (40.0)	195 (77.7)	

	IL-8		
	Low	High	p-value
	n (%)	*n* (%)	Chi^2
Gleason Score (*n*=256)			
2-6	58 (24.9)	1 (4.3)	*p*=0.0256
7-10	175 (75.1)	22 (95.7)	



	Par-4		
	Low *n* (%)	High *n* (%)	p-value Chi^2
Arg1 (*n*=256)			
Low	96 (67.6)	40 (35.1)	p<0.0001
High	46 (32.4)	74 (64.9)	
iNOS (*n*=256)			
Low	38 (26.8)	9 (7.9)	p=0.0001
High	104 (73.2)	105 (92.1)	
IL-8 (*n*=256)			
Low	128 (90.1)	105 (92.1)	p=0.5849
High	1 4 (9.9)	9 (7.9)	

Fig. 8. Arg1, iNOS and IL-8 expression in human PCa. TMAs containing 256 human adenocarcinoma and 35 normal matching prostate tissues were immunostained for the corresponding antibodies. Representative images of low and high intensity are shown.

Key research accomplishments

- Identification of Par-4 and PKCζ as new tumor suppressors that cooperate with PTEN.
- Characterization of a molecular network between Par-4 and PTEN on which the two tumor suppressors regulate each other's expression levels.
- Demonstration that simultaneous loss of Par-4 or PKCζ and PTEN promotes invasive PCa.
- Identification of NF-κB as a new molecular mechanism governing Par-4 and PTEN cooperation.
- Identification of the inflammatory cytokine IL-6 as an important novel marker of invasive PCa caused by simultaneous loss of the tumor suppressors Par-4 and PTEN.
- Chronic inflammation and increased recruitment of inflammatory cells is associated with invasive carcinoma in two different models: PTEN/Par-4 and PTEN/PKCζ
- PTEN/Par-4 immune system favors tumorigenesis, indicating that inflammation is not only secondary to PCa progression, but is also a key promoting factor.
- Par-4 is expressed in the stroma, and its loss impacts the TGF-b pathway.
- Par-4 and PTEN levels directly correlate in human PCa.
- IL-6 levels are inversely associated with Par-4 and PTEN levels in human PCa.
- Arg1 and INOS are highly overexpressed in the double mutants and their levels correlate with Par4 in human PCa.

Reportable outcomes

A) Personnel receiving funding from this research effort:

Shadi Abu-Baker, PhD (Univ. Cincinnati), Juan F. Linares, PhD and Ji-Young Kim, PhD (Sanford-Burnham Medical Research Institute).

B) Manuscripts published:

Fernandez-Marcos, P.J., Abu-Baker, S., Joshi, J., Galvez, A., Castilla, E.A., Canamero, M., Collado, M., Saez-Torres, C., Moreno-Bueno, G., Palacios, J., Leitges, M., Serrano, M., Moscat, J. and Diaz-Meco, M.T. (2009) Simultaneous inactivation of Par-4 and PTEN in vivo leads to synergistic NF-κB activation and invasive prostate carcinoma. Proc. Natl. Acad. Sci. USA. 106, 12962-12967 (Direct Submission)

Diaz-Meco, M.T.* and Abu-Baker, S. (2009) The Par-4/PTEN connection in tumor suppression. Cell Cycle 8(16), 2518-2522 (* corresponding author)

C) Reviews:

Moscat, J., Diaz-Meco, M.T., Wooten, M.W. (2009) Of the atypical PKCs, Par-4 and p62: Recent understandings of the biology and pathology of a PB1-dominated complex (Review) Cell Death and Differentiation 16(11), 1426-1437

Diaz-Meco, M.T., Moscat, J. (2012) The atypical PKCs in Inflammation: NF-κB and beyond (Review). Immunological Reviews 246(1):154-167

D) Abstracts:

"Cooperation between Par-4 and PTEN in Prostate Tumorigenesis". Maria T. Diaz-Meco, Pablo J. Fernandez-Marcos, Shadi Abu-Baker, Manuel Serrano and Jorge Moscat. FASEB Summer Research Conference "Lipid Signaling Pathways in Cancer" (Arizona, USA, 2009).

"Inflammation in prostate carcinogenesis: role of the tumor suppressor Par-4". Maria T. Diaz Meco, Shadi Abu-Baker, and Andrew Paluch. ImPaCT 2011 (Orlando, USA 2011)

E) Manuscripts in preparation:

"Role of Par-4-induced inflammation in prostate cancer". Shadi Abu-Baker, Ji-Young Kim, Juan F. Linares, and Maria T. Diaz-Meco. (2012)

"PKCz: a novel tumor suppressor in prostate cancer" Ji-Young Kim, Tania Valencia, Shadi Abu-Baker, Jorge Moscat and Maria T. Diaz-Meco (2012)

Conclusion

We have successfully completed this project. The results generated with this DOD award have allowed us to generate two new mouse models of prostate cancer progression and the identification of Par-4 and PKCζ as novel tumor suppressors in PCa. We have characterized the molecular mechanisms involved in the cooperation of PTEN and Par-4 and identified the inflammatory NF-κB pathway to be an important mediator of this process. Importantly, our results also demonstrate that inflammation is not only

secondarily associated to carcinogenesis, but it is an important contributing factor in tumor promotion. Furthermore, we identified iNOS and Arg1 as key events in the anti-tumoral response to maintain suppression of T cell function. In addition, we have validated our findings in human PCa samples. Of note, Par-4 expression correlates with PTEN levels and IL-6 expression levels display an inverse correlation with Par-4 and PTEN levels suggesting a critical role of inflammation in human PCa that could be of value as a biomarker.

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Appendices

Manuscripts published:

Fernandez-Marcos, P.J., Abu-Baker, S., Joshi, J., Galvez, A., Castilla, E.A., Canamero, M., Collado, M., Saez-Torres, C., Moreno-Bueno, G., Palacios, J., Leitges, M., Serrano, M., Moscat, J. and Diaz-Meco, M.T. (2009) Simultaneous inactivation of Par-4 and PTEN in vivo leads to synergistic NF-κB activation and invasive prostate carcinoma. Proc. Natl. Acad. Sci. USA. 106, 12962-12967 (Direct Submission)

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"Inflammation in prostate carcinogenesis: role of the tumor suppressor Par-4". Maria T. Diaz Meco, Shadi Abu-Baker, and Andrew Paluch. ImPaCT 2011 (Orlando, USA 2011)

Simultaneous inactivation of Par-4 and PTEN in vivo leads to synergistic NF-κB activation and invasive prostate carcinoma

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Prostate cancer is one of the most common neoplasias in men. The tumor suppressor Par-4 is an important negative regulator of the canonical NF-κB pathway and is highly expressed in prostate. Here we show that Par-4 expression is lost in a high percentage of human prostate carcinomas, and this occurs in association with phosphatase and tensin homolog deleted from chromosome 10 (PTEN) loss. Par-4 null mice, similar to PTEN-heterozygous mice, only develop benign prostate lesions, but, importantly, concomitant Par-4 ablation and PTEN-heterozygosity lead to invasive prostate carcinoma in mice. This strong tumorigenic cooperation is anticipated in the preneoplastic prostate epithelium by an additive increase in Akt activation and a synergistic stimulation of NF-κB. These results establish the cooperation between Par-4 and PTEN as relevant for the development of prostate cancer and implicate the NF-κB pathway as a critical event in prostate tumorigenesis.

AKT | aPKC | IL-6 | inflammation | prostate cancer

MEDICAL SCIENCES

Prostate cancer is one of the most common malignancies and the second leading cause of cancer death in males (1). The disease is complex in its development and response to therapy, and it cannot be predicted when or whether an indolent prostate tumor will become clinically aggressive. Significant limitations in current treatment methods warrant an intense focus on this type of cancer. Moreover, the development of targeted antitumor therapies will require a better understanding of the signaling cascades involved in the initiation and progression of prostate cancer.

Par-4 is a gene highly expressed in the prostate that was initially identified in an in vitro differential screen for proapoptotic genes in human prostate carcinoma cell lines (2). The *Par-4* gene maps to chromosome 12q21, a region frequently deleted in certain malignancies, and encodes a protein (38 kDa) containing a leucine-zipper domain in the carboxy-terminal region, which interacts with a variety of proteins (3), including the atypical protein kinases (aPKCs), PKCζ and PKCλ/ι (4). *Par-4* has been proposed to impair cell survival through the inhibition of the aPKCs and the consequent down-modulation of NF-κB and its prosurvival transcriptional targets (5–7). We have previously shown that the genetic inactivation of *Par-4* in mice leads to reduced lifespan and spontaneous tumorigenesis (6). Particularly relevant to this study, *Par-4*-null mice develop spontaneous benign neoplasias in hormone-dependent tissues, including prostate (6). In addition, we have also shown that *Par-4* is downregulated in ≈40% of human endometrial carcinomas and human lung adenocarcinomas (8, 9). Moreover, loss of *Par-4* dramatically increases Ras-induced lung carcinoma formation in association with enhanced NF-κB and Akt activity (9). The latter results unveiled an unanticipated role for *Par-4* as an indirect inhibitor of Akt, both in vitro and in vivo, through down-modulation of PKCζ (9). Together, these observations identify *Par-4* as a tumor suppressor in the NF-κB and Akt pathways in lung cancer (9).

The phosphatase and tensin homolog deleted from chromosome 10 (PTEN) tumor suppressor is a central regulator of human prostate carcinogenesis (10). PTEN alterations have been extensively implicated in human prostate cancer; PTEN deletions and mutations occur on at least 1 allele in up to 30% of primary cancers, and homozygous PTEN inactivation is frequently associated with metastatic prostate tissues (11, 12). In addition, loss of PTEN expression correlates with higher Gleason scores in human prostate cancer (13). PTEN encodes a lipid phosphatase that is a negative regulator of the PI-3K/Akt pathway (14) and, consequently, loss of PTEN function results in aberrant activation of the Akt pathway in prostate cells (14–16). In keeping with this, genetic ablation of Akt1 is sufficient to suppress tumor development in $PTEN^{+/-}$ mice (17). This relates to an emerging paradigm in cancer biology in which signaling activation is enhanced by the concomitant reduction of tumor suppressors acting in the same pathway, thus promoting tumor progression. For example, the tumor suppressor promyelocytic leukemia cooperates with PTEN inside the nucleus to inhibit Akt (18). In addition, PTEN loss synergizes with defects in a number of negative regulators of proliferation, such as Nkx3.1, p27, or p18INK4c to promote the progression of benign prostate tumors to invasive carcinoma (19–21). Consistent with this, transgenic expression of activated Akt in the murine prostate induces prostatic intraepithelial neoplasia (PIN) (22). However, Akt activation is not sufficient to drive this relatively benign form of neoplasia to more aggressive cancer phenotypes (22). This result suggests a 2-hit model for prostate tumor development involving the cooperation of complementary networks of tumor suppressors.

In this regard, signaling cascades other than Akt that are involved in the regulation of cell growth and survival could come into play during tumor progression. An important pathway is the NF-κB cascade, which appears to play a central role in carcinogenesis (23), although its implication in prostate cancer still needs to be better understood. Because Par-4 is a negative regulator of NF-κB (3, 7), and Par-4 loss leads to benign prostate neoplasias, we hypothesized that Par-4 deficiency in conjunction with the loss of an Akt inhibitor like PTEN could be instrumental in prostate cancer progression. Here we have investigated the cooperation between Par-4 and PTEN in prostate tumorigenesis and report that PTEN heterozygosity synergizes with Par-4 loss to promote the progression to

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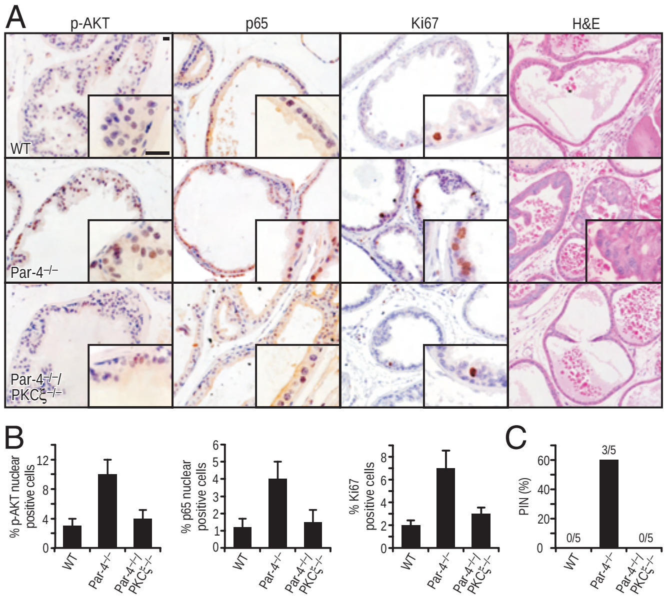

Fig. 1. Loss of PKCζ reverts the Par-4$^{-/-}$ phenotype in the prostate. Mice (4-week-old) of the indicated genotype were treated for 4 weeks with s.c. pellets containing testosterone (25 mg) and estradiol (2.5 mg). (*A*) Tissue sections were stained with anti-phospho-Akt(S473), anti-p65 (Rel A), or anti-Ki67 antibody, or H&E. Representative sections of analyzed prostate tissue after treatment are shown. $n = 5$ mice per genotype. (Scale bar, 50 μm.) (*B*) Quantification of cells showing positive nuclear staining for phospho-Akt, p65 or Ki-67. Results are the mean $\pm$ SD of counts from 10 different fields per mouse of a total of 5 mice in each condition. (*C*) Quantification of incidence of hormonally induced PIN lesions.

Table 1. Par-4 and PTEN association in human prostate carcinomas

	Par-4			
	Negative *n* (%)	Intermediate *n* (%)	High *n* (%)	*P*-value (Chi²)
PTEN ($n = 37$)				
Negative	4 (80)	3 (21.4)	2 (11.1)	
Intermediate	1 (20)	7 (50)	7 (38.9)	
High	0 (0)	4 (28.6)	9 (50)	0.021
Nuclear p65 ($n = 37$)				
Negative	1 (20)	5 (35.7)	13 (72.2)	
Intermediate	2 (40)	7 (50)	5 (27.8)	
High	2 (40)	2 (14.3)	0 (0)	0.037
IL-6 ($n = 37$)				
Negative	1 (20)	11 (78.6)	15 (83.3)	
Intermediate	3 (60)	1 (7.1)	2 (11.1)	
High	1 (20)	2 (14.3)	1 (5.6)	0.038
Gleason score ($n = 37$)				
2–6	1 (20)	5 (35.7)	12 (66.7)	
7–10	4 (80)	9 (64.3)	6 (33.3)	0.092

prostate carcinoma. We also show that there is a concomitant loss of Par-4 and PTEN in human prostate carcinomas, suggesting the existence of a pathologically relevant biochemical and functional cooperation between these 2 tumor suppressors impinging the Akt and NF-κB pathways.

Results

Par-4 Deficiency Alters the Prostate Epithelium and Leads to Neoplasia Through PKCζ. Recent results from our laboratory unveiled an important role for Par-4 in the control of NF-κB and Akt phosphorylation through PKCζ in lung tissue and in cell culture (9). Therefore, it would be of great interest to determine whether NF-κB and Akt are activated in Par-4-deficient preneoplastic prostates and whether that activation depends on PKCζ. It is also important to define the contribution of PKCζ to the induction of prostate neoplasia in *Par-4*$^{-/-}$ mice. To address these important questions, we analyzed the signaling pathways altered in preneoplastic prostates from *Par-4*$^{-/-}$ mice and also from *Par-4*$^{-/-}$/*PKCζ*$^{-/-}$ double-knockout mice. Interestingly, we found a reproducible increase in the activation of Akt in the prostate epithelial cells of *Par-4*$^{-/-}$ mice that is reverted in the *Par-4*$^{-/-}$/*PKCζ*$^{-/-}$ prostates (Fig. 1 *A* and *B*). These results are in keeping with our recently published data demonstrating a role for the Par-4/PKCζ module in the control of Akt in vivo (9), and extend its function to the prostate. Consistent with the proposed implication of Par-4 in NF-κB regulation, analysis of a set of well-established NF-κB-dependent transcripts revealed a reproducible increase in the levels of these mRNAs in Par-4$^{-/-}$ prostates as compared to WT controls [supporting information (SI) Table S1]. Of special relevance is IL-6, which has been shown to be an important inflammatory cytokine that plays a central role in various types of cancer (24). Consistent with this, RelA (p65) staining revealed increased NF-κB activation in Par-4-deficient preneoplastic prostates but not in the Par-4$^{-/-}$/PKC$\zeta$$^{-/-}$ prostates see (Fig. 1 *A* and *B*). Collectively, these results establish the Par-4/PKCζ complex as a bona fide regulator of the NF-κB and Akt pathways in prostate.

We next determined whether the loss of PKCζ could have an impact on the proliferation of Par-4-deficient preneoplastic prostate epithelial cells. Analysis of Ki67 expression, an indicator of proliferation, revealed an increase in the proliferative index of the prostatic epithelium in *Par-4*$^{-/-}$ as compared to WT controls. Importantly, this increase was dramatically reduced in the Par-4$^{-/-}$/PKC$\zeta$$^{-/-}$ prostates (see Fig. 1 *A* and *B*), which indicates that PKCζ channels signals downstream of Par-4 that are relevant to cell proliferation.

Based on the above findings, we next tested the hypothesis that PKCζ inactivation would prevent PIN induction by Par-4 deficiency. In this experiment, we treated WT, *Par-4*$^{-/-}$, and *Par-4*$^{-/-}$/*PKCζ*$^{-/-}$ mice (4 weeks old) for 4 weeks with a testosterone-estradiol mixture, provided continuously through s.c. pellets, as described previously (6), which is carcinogenic for mice with genetic alterations in tumor suppressors. After treatment, we determined the appearance of PIN in the prostates of these mice. Consistent with our previous observations (6), the hormonal treatment promoted the induction of PIN in *Par-4*$^{-/-}$ mice, but not in WT mice. Interestingly, the phenotype of the *Par-4*$^{-/-}$/*PKCζ*$^{-/-}$ mice was, in this respect, the same as the WT mice, showing no PIN induction (Fig. 1*C*). Collectively, these results genetically demonstrate that PKCζ is essential for the hyperproliferation and development of prostate neoplasia triggered by Par-4 deficiency.

Loss of Par-4 Expression in Human Prostate Carcinomas. To determine the relevance of Par-4 as a prostate tumor suppressor, human prostate carcinomas were analyzed for Par-4 expression and promoter methylation. Immunohistochemical (IHC) analysis revealed that prostate carcinomas ($n = 41$) could be classified between Par-4-negative/low (59%) and Par-4-positive (41%) (Fig. S1*A* and Tables S2 and S3). Similar to what we described earlier for endometrial cancer (8), there was a significant association between Par-4 promoter methylation and lack of Par-4 expression (see Fig. S1 and Tables S2 and S3). Small cores from these tumors were assembled in a tissue microarray and subsequently analyzed by IHC for Par-4 and PTEN. Interestingly, a significant direct correlation between Par-4 and PTEN protein levels was observed (Table 1 and Fig. 2). The majority of Par-4-positive tumors were also positive for PTEN, and conversely, those tumors with negative/low PTEN also had negative/low Par-4 (see Table 1). This concomitant loss of Par-4

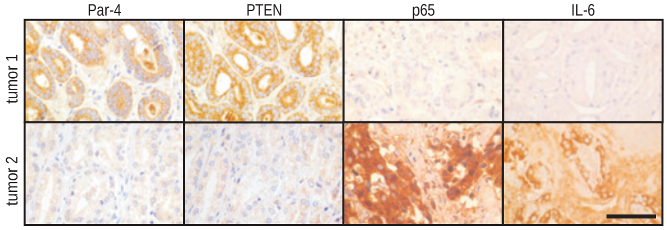

Fig. 2. Association between Par-4 and PTEN expression in human prostate cancer. Immunostaining for Par-4, PTEN, p65, and IL-6 in human prostate cancer samples. Representative examples of a tumor positive for both Par-4 and PTEN (tumor 1; *Upper*) and of a tumor negative for both Par-4 and PTEN (tumor 2; *Lower*). Par-4 and PTEN levels are inversely correlated with 2 parameters of NF-κB activation: nuclear p65 and IL-6. (Scale bar, 200 μm.)

and PTEN suggests the existence of cooperation between these 2 tumor suppressors. PTEN inactivation has previously been found to be associated with higher Gleason scores and to more aggressive prostate cancer. Similarly, we observed a correlation between Par-4 loss and high Gleason scores (see Table 1). Together, these results place Par-4 loss as a relevant step in prostate tumor progression, like PTEN deficiency, and reveal that Par-4 inactivation is at least in part usually achieved by aberrant de novo methylation of its promoter.

Simultaneous Deficiency in Par-4 and PTEN Promote Prostate Adenocarcinoma. As the loss of Par-4 is associated with PTEN deficiency in human prostate cancer (see Table 1) and increased Akt activity in preneoplastic murine prostates (see Fig. 1), we reasoned that complete deletion of Par-4 in the context of PTEN heterozygosis should lead to more aggressive prostate lesions. To address this question, we crossed *Par-4*$^{-/-}$ mice with *PTEN*$^{+/-}$ mice to generate *PTEN*$^{+/-}$/*Par-4*$^{+/-}$ and *PTEN*$^{+/-}$*Par-4*$^{-/-}$ mice. Total or partial ablation of Par-4 in mice in a PTEN-heterozygous genetic background did not significantly affect their overall survival (Fig. S2*A*), which was mainly determined by the development of multicentric lymphoproliferative disease. This suggests that *Par-4* gene dosage does not impinge on the latency or mortality associated with PTEN-driven lymphoproliferative disease. Importantly, however, the loss of *Par-4* does cooperate with PTEN-heterozygosity in prostate cancer. Thus, whereas the majority of prostate glands in *Par-4*$^{-/-}$ mice were normal, with only a few glands becoming mildly hyperplastic in mice older than 12 months of age (data not shown), *Par-4* deficiency in a *PTEN*$^{+/-}$ background had an impact on cancer initiation, increasing the incidence of low-grade PIN (PIN I) lesions in a manner dependent on the *Par-4* gene dosage (Fig. 3*A*). Interestingly, Par-4 also cooperates with PTEN heterozygosity in the progression to high-grade lesions. Thus, whereas at 6 months of age the prostates of *PTEN*$^{+/-}$ mice were free of high-grade PIN (PIN III and PIN IV) (see representative lesions in Fig. S2*B*), the prostates of *PTEN*$^{+/-}$/*Par-4*$^{+/-}$ mice developed high-grade PIN at 100% penetrance (not shown). Consistently, the proliferative index of the prostate epithelium, measured as the percentage of nuclei positive for Ki67, correlated with PIN progression (Fig. 3 *B* and *C*). Of note, the absence of Par-4 in a PTEN$^{+/-}$ background led to enhanced survival of neoplastic cells (Fig. 3 *D* and *E*). These are important observations because, whereas the oncogenic coopera-

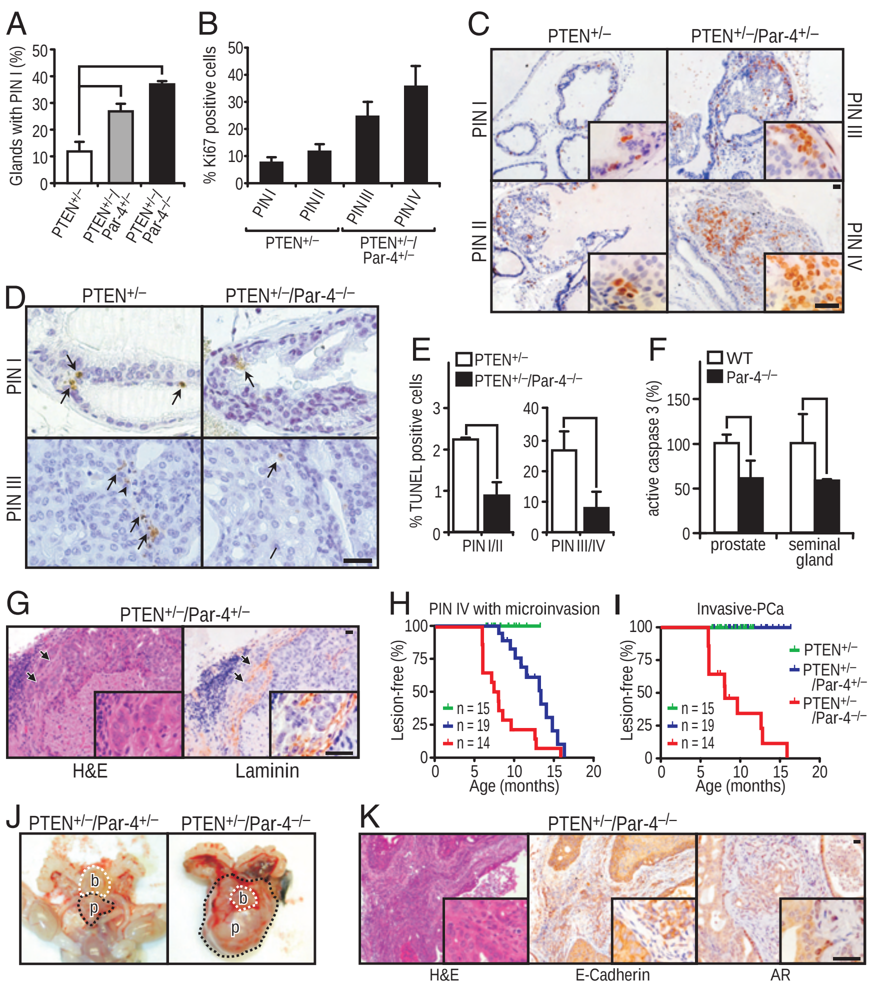

Fig. 3. Loss of Par-4 cooperates with PTEN$^{+/-}$ to promote invasive prostate carcinoma. (*A*) Par-4 status affects prostate cancer initiation and low grade PIN (PIN I) is increased depending on *Par-4* gene dosage. $n = 5$ mice per genotype. *, $P < 0.05$; **, $P < 0.01$. (*B* and *C*) The proliferative index (%Ki67 positive) correlates with PIN grade. Representative Ki67 staining of prostates from PTEN$^{+/-}$ and PTEN$^{+/-}$/Par-4$^{+/-}$ mice at 6 months of age is shown. (*D* and *E*) Apoptosis was measured in PINs by TUNEL in mice of the indicated genotype ($n = 3$ per genotype). Representative TUNEL stainings are shown (*arrows* in *D*). At least 15 PINs of each group (PIN I/II or PIN III/IV) were measured for each genotype at 8 months of age. Values correspond to the number of apoptotic cells per lesion. *, $P < 0.05$. (*F*) Apoptosis, measured as active caspase 3-positive areas, in prostates of mice of the indicated genotypes ($n = 4$ per genotype) 3 days after castration. *, $P < 0.05$; **, $P < 0.01$. (*G*) Representative H&E staining of microinvasive carcinoma associated with high-grade PIN in the dorsolateral area of a 12-month-old PTEN$^{+/-}$/Par-4$^{+/-}$ mouse (*Left*). Laminin immunostain demonstrates penetration of small nests (*arrowheads*) through the basement membrane into the surrounding stroma. $n = 5$ mice per genotype (*Right*). (*H* and *I*) Kaplan-Meier curves of incidence of advanced prostate intraepithelial neoplasia (PIN IV) with microinvasion (*H*) or of invasive prostate carcinoma (*I*). A clear correlation between the progression of prostate tumorigenesis and loss of 1 or 2 *Par-4* alleles in combination with PTEN heterozygosity was observed. (*J*) Representative examples of macroscopic appearances of age-matched PTEN$^{+/-}$/Par-4$^{+/-}$ and PTEN$^{+/-}$/Par-4$^{-/-}$ mouse organs at 12 months of age are shown. Significant enlargement of prostate (p) was seen; (b) denotes the bladder. (*K*) Representative examples of invasive carcinoma in the dorsolateral area of a 6-month-old PTEN$^{+/-}$/Par-4$^{-/-}$ mouse. Staining methods include H&E, E-cadherin, and androgen receptor (AR). $n = 5$ mice per genotype. (Scale bar = 50 μm.)

tion between PTEN deficiency and other tumor suppressors, such as Nkx3.1, p18, p27, p53 or Tsc2, has been assigned mainly to an increase in proliferation (20, 25), Par-4 deficiency is unique in the sense that it involves an increase in both survival and proliferation. In keeping with this, Par-4 deletion decreased castration-induced apoptosis in the prostates and seminal gland of Par-4-deficient mice, further demonstrating the important role of Par-4 in the control of prostate cell survival (Fig. 3*F*).

Interestingly, the high-grade PIN lesions progressed to microinvasive carcinoma in the PTEN$^{+/-}$/Par-4$^{+/-}$ double heterozygotes (Fig. 3 *G* and *H*), as evidenced by disruption of the basal membrane of the epithelium identified by laminin staining and the presence of small nests of cells invading the surrounding stroma (see Fig. 3*G*). More importantly, the synergy in the progression to prostate carcinoma depends on *Par-4* in a gene-dosage-dependent manner, as the loss of both *Par-4* alleles in the context of PTEN$^{+/-}$ promoted fully invasive carcinoma with an onset at 6 months of age, high penetrance, and dramatically enlarged prostates (Fig. 3 *I* and *J*). Invasive prostate carcinomas recapitulated the aggressive features of human prostate cancer, such as invasion, foci of highly anaplastic cells, vascular emboli, and a solid pattern of growth (Fig. S2*C*). Moreover, high-grade PIN lesions were observed in all 3 lobes (anterior, ventral, and dorsolateral), while invasive prostate carcinoma occurred predominantly in the dorsolateral prostate. To define the origin of carcinomas in the double-mutant prostates, we performed IHC analyses of various markers. As shown in Fig. 3*K*, PTEN$^{+/-}$Par-4$^{-/-}$ epithelial cancer cells were positive for E-cadherin staining and expressed high levels of androgen receptor, a hallmark of secretory epithelium, but were negative for the neuroendocrine cell marker synaptophysin (Fig. S2*D*). These results indicate the epithelial origin of the invasive carcinoma developed as a result of the loss of Par-4 in the context of PTEN heterozygosity. Together, these observations indicate that Par4-deficiency has a profound impact on prostate tumorigenesis in association with PTEN-deficiency, affecting the number, size, progression, and severity of lesions from benign intraepithelial neoplasias to aggressive carcinomas in a manner dependent on *Par-4* gene dosage.

Akt and NF-κB Activation in PTEN$^{+/-}$/Par-4$^{-/-}$ Prostate Epithelium. Because both Par-4 and PTEN are negative regulators of Akt [(9, 10), see also Fig. 1], we predicted that the inactivation of Par-4 in a PTEN$^{+/-}$ background would lead to an even greater increase in Akt activity, as compared to that induced in the single-mutant prostates. To address this possibility, we determined Akt activity in preneoplastic prostates from mice of different genotypes by IHC with anti-pAkt antibody. Results in Fig. 4 *A* and *B* demonstrate that the total loss of Par-4 in a PTEN heterozygous background results in more nuclear pAkt staining than in WT or single-mutant prostates. However, compared to the single mutants, the increase in pAkt in the *PTEN$^{+/-}$/Par4$^{-/-}$* double mutants is only additive and, therefore, difficult to reconcile with the observed dramatic effect on tumor onset and progression.

In this regard, it is well established that Par-4 negatively controls NF-κB (7). Thus, we sought to determine whether the simultaneous mutation of Par-4 and PTEN would lead to a synergistic activation of NF-κB in preneoplastic prostates. To address this question, we stained these prostates with an anti-p65 (RelA) antibody and scored for nuclear translocation of p65 (RelA), an established marker of NF-κB activation. While Par-4 or PTEN insufficiency separately gave rise to a significant, although modest, activation of NF-κB, this parameter was dramatically activated in the double-mutant preneoplastic prostates, suggesting a synergistic effect of the 2 mutations for NF-κB activation (see Fig. 4 *A* and *B*). When other parameters of this pathway were assessed, such as IKK activation, measured as phospho-IKK levels, or the phosphorylation of p65, a similar synergistic response was observed (see Fig. 4 *A* and *B* and Fig. S3). This was consistent with a synergistic increase in mRNA levels of NF-κB target genes, such as Kc, IL-6, TNF-α, and Fhc in

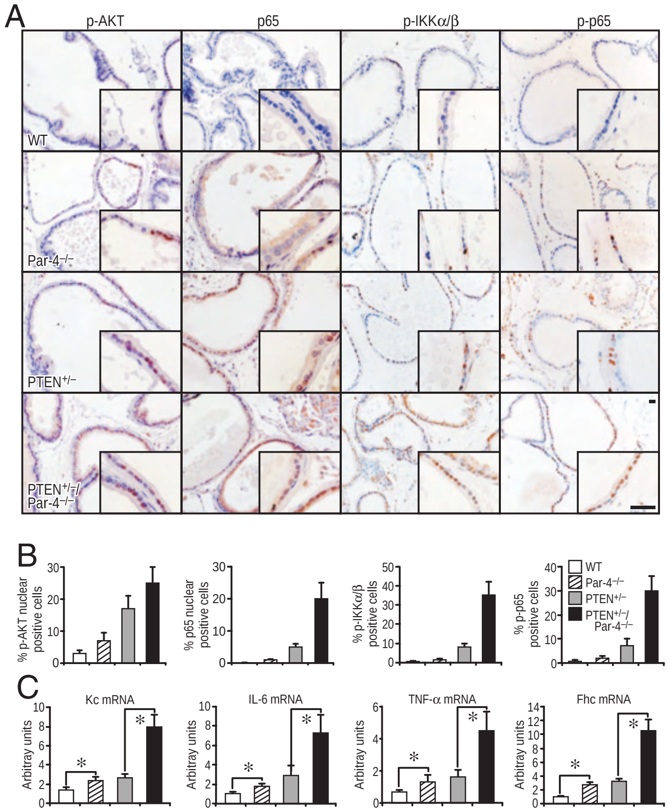

Fig. 4. Par-4 and PTEN deficiencies cooperate in Akt and NF-κB activation in preneoplastic prostate. (*A*) Representative sections of preneoplastic prostate glands from 6-month-old mice of the indicated genotypes are shown. Tissue sections were stained with anti-phospho-Akt(S473) anti-p65 (Rel A), anti-phospho-IKKα/β (S176/180), or anti-phospho-p65 (S276) antibody. (Scale bar, 50 μm.) (*B*) Quantification of cells showing positive staining for each antibody. Results are the mean ± SD of 10 different fields per mouse of a total of 5 mice in each condition. (*C*) Quantification by qRT-PCR of mRNA levels of the following NF-κB targets: Kc, IL-6, TNF-α, and Fhc. Results are shown as mean ± SD. *, $P < 0.05$.

the double mutants as determined by qRT-PCR (Fig. 4*C*), and is in agreement with the enhanced IL-6 levels observed in preneoplastic prostate of the double mutant mice as compared to the single mutants (see Fig. S3). This suggests that the synergistic activation of NF-κB in the doubly mutant prostate epithelium is the critical event for tumor progression in this system.

To confirm the relevance of NF-κB activation in the cooperation of Par-4 and PTEN mutations in human prostate cancer, we studied in the same human tissue microarray samples used above whether the expression levels of Par-4 and PTEN correlate with activated NF-κB, as determined by nuclear p65 (Rel A) and IL-6 IHC staining. Importantly, we found that decreased levels of PTEN and Par-4 were significantly associated with an increase in both nuclear p65 and IL-6 expression, as well as with disease progression (see Fig. 2 and Table 1). These results demonstrate that the simultaneous inactivation of both tumor suppressors lead to increased activation of the NF-κB pathway in human prostate cancer tumors.

Cell Autonomous Cooperation Between Par-4 and PTEN Deficiencies. We next investigated whether the synergistic effect of simultaneous PTEN and Par-4 deficiencies detected in prostate tumorigenesis is a cell-autonomous phenomenon that can be generalized to other cell types. We first determined the proliferative properties of Par-4-deficient immortalized embryonic fibroblasts (EFs) in which PTEN levels were knocked down by lentiviral shRNA (PTENi).

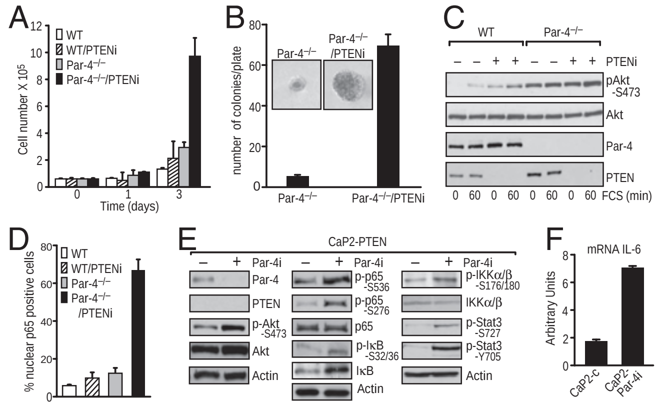

Fig. 5. Synergistic proliferation, transformation, and NF-κB activation in cell cultures. (*A*) WT and Par-4$^{-/-}$ EFs were retrovirally infected with control shRNAi or an shRNAi specific for PTEN (PTENi), and growth curves were determined at different times. (*B*) Colony formation in soft agar from Par-4$^{-/-}$ EFs infected with control shRNAi or PTEN shRNAi (PTENi). The total number of colonies per plate was scored by counting and represented as mean ± SD of 6 plates from 2 independent experiments; representative picture showing the colony size difference (*Inset*). (*C*) Akt activation in response to serum (FCS) was increased in Par-4$^{-/-}$ EFs treated with PTEN shRNAi. EF extracts were analyzed with the different antibodies (as labeled) by immunoblotting. (*D*) NF-κB activation analyzed by immunofluorescence staining with anti-p65 (Rel A). The percentage of p65 nuclear-positive cells was determined by counting 10 fields for each experimental condition. Results are the mean ± SD. (*E*) Knockdown of Par-4 (Par-4i) was achieved by lentiviral infection in the prostatic epithelial cell line, PTEN-CaP2, and cell extracts were analyzed by immunoblotting with the different antibodies (as labeled). (*F*) qRT-PCR analysis of IL-6 mRNA levels in PTEN-CaP2 cells infected with control or Par-4 lentivirus (Par-4i). Results are shown as mean ± SD.

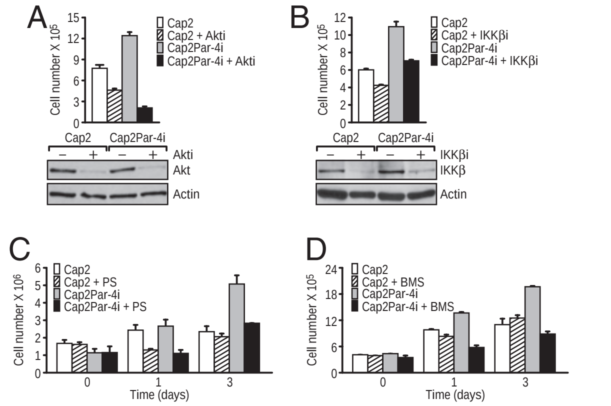

Fig. 6. Functional contribution of the NF-κB pathway in Par-4 and PTEN cooperation. (*A* and *B*) CaP2 or CaP2-Par-4i cells were treated with a control siRNA or with siRNAs specific for Akt (*A*) or IKKβ (*B*) and cell viability was determined by Trypan Blue exclusion 72 h after transfection. Cell extracts were analyzed in parallel by Western blot with antibodies for Akt, IKKβ, and actin (*Lower*). Results are the mean ± SD of triplicates. (*C* and *D*) Effect of treatment of CaP2 or CaP2-Par-4i cells with 2 inhibitors of the NF-κB pathway: PS1145 (10 μM) or BMS-345541(1 μM).

The data in Fig. 5*A* demonstrate that cell proliferation was not greatly affected by the loss of Par-4 alone or by the down-regulation of PTEN. However, the simultaneous inactivation of both proteins dramatically enhanced cell proliferation, indicating a cell-autonomous and synergistic interaction between the 2 proteins. Results in Fig. 5*B* reinforce this notion, as Par-4-null/PTENi cells were able to form colonies in soft agar, whereas Par-4-deficient (see Fig. 5*B*) or PTENi (not shown) cells could not. The data in Fig. 5*C* show that PTEN depletion was efficient in the RNAi-infected cells. Fig. 5*C* also shows that Par-4 deficiency or PTEN knockdown increased pAkt levels in EFs, as expected. In addition, pAkt levels in the doubly deficient cells revealed an additive increase in Akt activity. Of great relevance from the point of view of our in vivo data shown in Fig. 4, the nuclear translocation of p65 (Rel A) in the double Par-4$^{-/-}$/PTENi EFs synergistically increased as compared to that in Par-4$^{-/-}$ or PTENi cells (Fig. 5*D*). Taken together, these results demonstrate that the simultaneous inactivation of Par-4 and PTEN in vivo or in vitro leads to synergistically increased NF-κB levels in vivo in a cell-autonomous manner.

To further reinforce the above concept, we used the murine PTEN-null CaP2 prostate epithelial cell line (26). The knockdown of Par-4 by shRNA (Par-4i) lentiviral infection in these cells led to a detectable increase in pAkt as compared to control cells (Fig. 5*E*, *Left*), and more interestingly, to a robust activation of NF-κB (determined as phosphorylation of p65, IκB, and IKK) in the Par-4 knockdown cells as compared to the control cell line (see Fig. 5*E*, *Middle* and *Right*). Consistent with this, levels of IL-6 mRNA, a bona fide NF-κB-dependent gene, were also significantly activated in Par-4 knockdown cells (Fig. 5*F*). Interestingly, this enhanced expression of IL-6 in CaP2-Par-4i cells correlated with a higher level of Stat3 phosphorylation in these samples (see Fig. 5*E*, *Right*), in keeping with the notion that the IL-6 produced under these conditions is biologically active. These results suggest that the cooperation between Par-4 and PTEN is a cell-autonomous effect and that the activation of Akt and NF-κB are important contributors to their mechanism of action.

Functional Relevance of the NF-κB Pathway to the Mechanism of Action of Par-4 and PTEN Mutations. To demonstrate the functional contribution of activation of the Akt and NF-κB pathways in vivo, we tested whether the knockdown of Akt or IKKβ was sufficient to block the enhanced cell proliferation observed in the CaP2-Par-4i cells. Results in Fig. 6 *A* and *B* show that both pathways contribute to the proliferative advantage of CaP2-Par-4i cells, as siRNAs for Akt and IKK significantly inhibit this effect. To further test the functional role of the NF-κB activation in this system, we used 2 well-characterized highly specific small-molecule IKK pharmacological inhibitors, PS1145 and BMS-345541 (27, 28). Interestingly, treatment of CaP2-Par-4i cells with these molecules dramatically blocked cell growth in these cells (Fig. 6 *C* and *D*), consistent with the IKKβ sRNAi experiment. This suggests that activation of both pathways is responsible for the cooperative effect of Par-4 and PTEN inactivation in cell cultures in vitro, and it most likely accounts for the in vivo phenotype of the double mutant prostates.

Discussion

The role of Par-4 in Akt regulation, and the fact that it is highly expressed in prostate (2, 9), opened the possibility that Par-4 could be a player in prostate cancer, as the Akt pathway has been shown to be relevant in this type of neoplasia in both mice and humans (29). Interestingly, we report here a frequent loss of Par-4 in human prostate cancers, often associated with aberrant promoter methylation, a situation reminiscent of what we have reported before in human endometrial cancer (8). Moreover, we observed a correlation between Par-4 and PTEN loss, as well as between Par-4 deficiency and high Gleason score. Taken together, this suggests that loss of the 2 tumor suppressors has a strong cooperative effect on human prostate carcinogenesis. This observation led us to reason that ablation of Par-4 in the context of PTEN heterozygosity should lead to aggressive forms of prostate cancer. The data we show here demonstrate that, in fact, this is the case, because the inactivation of Par-4 in the context of PTEN heterozygosity resulted in invasive adenocarcinoma that correlates with the additive activation of Akt and the synergistic stimulation of NF-κB in preneoplastic prostates. These 2 signaling cascades, NF-κB and Akt, have been mostly implicated in cell survival and, accordingly, we ob-

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served in castration experiments and TUNEL analysis that Par-4 deficiency has an important effect on the survival of prostate epithelial cells. This appears to be a proprietary characteristic of Par- 4 deficiency because it has not been reported for other tumor suppressors in combination with PTEN deficiency (20, 21, 25).

Notably, previous studies have demonstrated that both pathways, Akt and NF-κB, are deregulated in prostate cancer. In this regard, the activity of NF-κB is higher in androgen-independent cell lines and xenografts, as well as in metastatic prostate cancer compared with localized disease (30). Furthermore, increased NF-κB activation correlates with poor prognosis and predicts relapse (31). On the other hand, deregulated expression and mutations of PTEN occur with high frequency in prostate cancer, leading to aberrant activation of Akt and its downstream targets (29). However, not all of the phenotypes associated with PTEN loss can be fully explained by the activation of Akt (32), and it is increasingly apparent that PTEN possesses functions that are independent of its ability to suppress PI3K (32). Therefore, it is possible that PTEN deficiency in the context of cooperation with the inactivation of other tumor suppressors may set in place new molecular mechanisms to promote prostate tumorigenesis and progression. Our data demonstrate the existence of such a unique mechanism involving the synergistic induction of NF-κB because of the simultaneous inactivation of Par-4 and PTEN. Moreover, this effect is cell-autonomous and can be recapitulated in cultures of EFs and epithelial prostate cancer cells. Of note, blockade of the NF-κB pathway with an IKKβ sRNAi or with small-molecule inhibitors reverted the proliferative advantage of PTEN- and Par-4-deficient cells. More importantly, and consistent with these results, our IHC analysis of a set of human prostate cancer tumors showed that the loss of PTEN and Par-4 expression correlated with increased activation of the NF-κB pathway. An interesting aspect of these studies is that inactivation of Akt in these cell cultures also impaired cell proliferation in the Par-4/PTEN doubly inactivated cells, even though its activation in this context was not synergistic. These results could be interpreted to mean that Akt is necessary, but might not be sufficient, to drive the fully invasive prostate cancer phenotype, which would require the synergistic activation of NF-κB. Consistent with this notion are previously published observations that the transgenic expression of activated Akt in prostate is not sufficient to drive the invasive phenotype (22). Data from Baldwin's laboratory suggest the existence of a link between Akt and NF-κB in PTEN-deficient prostate cancer cells (33). Future studies should address the potential role of Akt as a permissive step in the synergistic activation of NF-κB during prostate cancer progression from a benign phenotype to more invasive stages.

In summary, our data establish a unique paradigm whereby Par-4 and PTEN mutations show accelerated tumor progression through the cooperation of the loss of these tumor suppressors in prostate carcinogenesis by the activation of the Akt and NF-κB cascades. Therefore, the use of NF-κB inhibitors alone or in combination with PI3K/Akt targeted molecules might be a promising therapeutic strategy in prostate cancers where both tumor suppressors have been inactivated.

Materials and Methods

Mice. *Par-4*$^{-/-}$, *PKC*$\zeta^{-/-}$, and *PTEN*$^{+/-}$ mice were described previously (7, 34, 35). All mice were born and kept under pathogen-free conditions. Animal handling and experimental procedures conform to institutional guidelines (University of Cincinnati Institutional Animal Care and Use Committee, and Guidelines for Humane Endpoints for Animals Used in Biomedical Research at the Spanish National Cancer Research Center). All genotyping was done by PCR.

Histological Analysis. Prostates were dissected and fixed in 10% neutral buffered formalin for 24 h, dehydrated, and embedded in paraffin. Sections (5 μm) were cut and stained with H&E. An extended section of *Materials and Methods* is provided in the *SI Materials and Methods*.

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Extra View

The Par-4/PTEN connection in tumor suppression

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Abbreviations: Akt (PKB), protein kinase B; aPKC, atypical PKC; DLK, dual leucine zipper kinase; ERG, ETS related gene; FGF8b, fibroblast growth factor 8, isoform b; IL-6, interleukin-6; IL-8, interleukin-8; mTOR, mammalian target of rapamycin; MUK, NFκB, nuclear factor κB; Par-4, prostate apoptosis response-4; PCa, prostate cancer; PI 3-kinase, phosphoinositide 3-kinase; PIN, prostatic intraepithelial neoplasia; PIP, phosphatidylinositol; PKC, protein kinase C; PML, promyelocytic leukemia; PTEN, phosphatase and tensin homolog gene; Rb, retinoblastoma; Rheb, Ras homolog-enriched in brain; TNFα, tumor necrosis factor α; Tsc2, tuberous sclerosis complex 2; WT1, Wilms' tumor 1; XIAP, X-linked inhibitor of apoptosis protein; ZPK, zipper protein kinase

Key words: Par-4, PTEN, aPKC, PKCζ, Akt, NFκB, prostate cancer, tumor suppressors

Tumor suppressors function in a coordinated regulatory network, and their inactivation is a key step in carcinogenesis. The tumor suppressor Par-4 is a novel integral player in the PTEN network. Thus, Par-4 is absent in a high percentage of human prostate carcinomas, and its loss is concomitantly associated with PTEN loss. Genetic ablation of Par-4 induces fully invasive prostate carcinomas in PTEN-heterozygous mice. In contrast, Par-4 deficiency alone, like PTEN heterozygosis, results in lesions that are unable to progress beyond the benign neoplastic stage known as PIN. At this PIN transition, the mutual induction of Par-4 and PTEN is an additional regulatory step in preventing cancer progression. Par-4 deficiency cooperates with PTEN haploinsufficiency in prostate cancer initiation and progression and their simultaneous inactivation, in addition to enhancing Akt activation, sets in motion a unique mechanism involving the synergistic activation of NFκB. These results suggest that the concurrent interruption of complementary signaling pathways targeting PI3K/Akt and NFκB activation could provide new and effective strategies for cancer therapy.

Introduction

Prostate cancer (PCa) is one of the most common malignancies in men. The prevalence of PCa is on the increase in western societies. It is among the leading causes of male cancer-related morbidity and death, second only to lung cancer, representing approximately 10% of all cancer deaths among men in the United States. Indeed, one in six men in the United States will be diagnosed with PCa during their lifetime. PCa is a complex disease in its development and response to therapy.

PCa proceeds through a series of defined steps, including prostatic intraepithelial neoplasia (PIN), invasive cancer, and hormone-dependent or hormone-independent metastasis. All these different stages have been well defined histologically, although the molecular mechanisms contributing to the initiation and progression of PCa are not fully understood. Diagnosis is based mainly on histology and Gleason scoring and, while effective for disease identification and determining general prognosis, these tools have limited usefulness in deciding the best course of treatment for patients with intermediate grade tumors.[1] Treatment is further complicated by the fact that prostate cancer initially responds well to androgen-ablation or anti-androgen therapy, but eventually enters an androgen-independent stage with no effective therapy.[2] Therefore, the development of new therapies and better diagnostic techniques will depend on increasing our understanding of the molecular basis of this disease.

Genetic loss or mutation of tumor suppressor genes is a frequent event initiating and/or promoting tumorigenesis.[3] The tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) is an important player in human prostate carcinogenesis and, with p53, represents one of the most frequently mutated genes in PCa. PTEN maps to 10q23, a locus that is highly susceptible to mutation in primary human cancers[4,5] and is commonly lost in metastatic prostate cancer.[6] Functionally, PTEN is a plasma-membrane lipid phosphatase that antagonizes the PI-3K/Akt pathway[7,8] by hydrolyzing phosphatidylinositol 3,4,5-trisphosphate (PIP_3) to generate phosphatidylinositol 4,5-trisphosphate PIP_2. Upon PTEN loss, PIP_3 accumulates and promotes the recruitment to the membrane of pleckstrin homology domain-containing proteins, including AKT, and their subsequent activation. Activation of Akt through deregulated PI3K signaling, resulting from genetic inactivation of PTEN or an activating mutation in PI3K, is a frequent molecular event in human cancer and one of the major signaling pathways implicated

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in advanced PCa.[9-12] Consistent with this, genetic inactivation of Akt1 suppresses tumor development in PTEN$^{+/-}$ mice. However, transgenic expression of activated Akt in the murine prostate is not sufficient to promote the development of invasive PCa, suggesting that Akt-independent pathways or the cooperation of complementary networks of tumor suppressors might be required for tumor progression.[13] In keeping with this, PTEN haploinsufficiency has been shown to promote cell proliferation and the development of PIN, but these lesions do not progress to invasive disease[14] unless there is a concomitant loss of other tumor suppressors. This opens an important avenue of study that involves the identification of novel tumor suppressors and the elucidation of the means by which they coordinate with each other to form an intricate regulatory network to protect against tumorigenesis.

The Tumor Suppressor Par-4 at the Crossroad of NFκB and Akt Signaling

Par-4 is a tumor suppressor originally identified in an in vitro differential screen of prostate cancer cells undergoing apoptosis following androgen withdrawal.[15] The Par-4 gene maps to chromosome 12q21, a region frequently deleted in certain malignancies.[16] Par-4's role as a tumor suppressor matches its tissue distribution in that it appears to be active in tissues in which is highly expressed such as prostate, endometrium and lung.[17,18] Consistent with this, in vivo studies of Par-4 KO mice show reduced lifespan, enhanced benign tumor formation, and low-frequency carcinogenesis. Par-4-deficient mice develop increased benign neoplasia in hormone-dependent tissues and cooperate with Ras to induce lung carcinoma in vivo. In addition, it has been shown that Par-4 is downregulated in approximately 40% of human endometrial carcinomas, human prostate carcinomas, and human lung adenocarcinomas.[17,19,20]

The Par-4 gene encodes a protein that harbors a leucine-zipper domain in the carboxy-terminal region, which interacts with several proteins including the atypical PKCs (aPKCs), the Wilms' tumor 1 (WT1) protein, and the kinase MUK/DLK/ZPK.[21,22] The interaction with WT1 and MUK/DLK/ZPK points to a nuclear role for Par-4 as a transcriptional repressor; however, in vivo genetic evidence supporting this function is sorely lacking. The available data based on genetic evidence support a model according to which the direct binding of Par-4 to the zinc-finger domain of both aPKC isoforms, PKCζ and PKCλ/ι, results in inhibition of their enzymatic activity.[23] This leads to the subsequent impairment of NFκB activation, as both aPKCs are relevant pro-inflammatory molecules for the regulation of the NFκB pathway.[24,25] In fact, multiple studies independently demonstrated that overexpression of Par-4 leads to inhibition of NFκB, thus potentiating TNFα-induced cell death.[26-28] In this regard, the loss of Par-4 in embryo fibroblasts leads to the hyperactivation of PKCζ and of NFκB transcriptional activity.[29] Consistent with this, the NFκB-dependent anti-apoptotic protein XIAP is expressed at significantly elevated levels in Par-4-null cells, which correlates with reduced caspase-3 activation and apoptosis.[29] In addition, Par-4 deficiency is associated with increased NFκB activation in both lung and prostate cells. Moreover, this hyperactivation is reversed upon loss of PKCζ in Par-4/PKCζ DKO mice, suggesting that PKCζ is a bona fide target of Par-4 in vivo. Interestingly, Par-4-deficient mice also have higher levels of activated Akt in lung and prostate epithelial cells, and, as is the case for NFκB, this activation is mediated by PKCζ.[17] Akt is a direct substrate of PKCζ at the phosphorylation site Ser124, which helps to control basal Akt activity by allowing the efficient phosphorylation of Akt at two other critical residues, Thr308 and Ser473, which are required for full activation.[17] This places Par-4 as a common step in the regulation of the Akt and NFκB pathways. A question that deserves further investigation is whether or not Akt is involved in the regulation of NFκB by the Par-4/PKCζ cassette.

Par-4, a Novel Tumor Suppressor in the PTEN Network

There are two primary ways that tumor suppressors could coordinate their activities in a regulatory network: they could impinge on a single signaling pathway to increase a required molecular threshold, or they could activate different complementary and downstream pathways that interact to create a synergistic effect. In this regard, a common mechanism of action underlying the cooperation of PTEN with other tumor suppressors is through the modulation of Akt activation. For example, the tumor suppressor PML cooperates with PTEN inside the nucleus to inhibit Akt through its recruitment and inhibition by the phosphatase PP2a in the PML nuclear bodies.[30] Another tumor suppressor, NEP, cooperates with PTEN through synergistic inhibition of the PI3K/Akt pathway by direct interaction of and stabilization of PTEN.[31] In addition, PTEN synergizes with other tumor suppressors, such as NKx3.1, p18 and Tsc2, through cooperation in Akt activation.[32-35] However, in addition to Akt activation, there could be other complementary mechanisms set in motion by the cooperative loss of PTEN and other tumor suppressors that have an important impact on the progression to invasive carcinoma. In fact, PTEN cooperates with Rb and p18 in a complementary collaboration through their role in controlling cell cycle progression.[33] PTEN haploinsufficiency cooperates with the overexpression of Rheb, an upstream activator of mTOR complex 1 and with the overexpression of FGF8b, a commonly occurring genetic aberration of human PCa, impinging on different signaling pathways.[36,37] Also, two recent studies show that the common recurrent gene fusion between TMPRSS2 and ERG promotes PCa when PTEN is concurrently lost.[36,38] ERG can act together with PTEN by inducing the transcription of downstream checkpoint genes that would usually be blocked by Akt and have a crucial role on cell migration and invasion. Such collaboration could provide a selective advantage at the cellular level to allow benign lesions to progress to cancer.

Par-4 is a newly identified player in the network of tumor suppressors that cooperate with PTEN (Fig. 1). Recent studies from our laboratory demonstrate that the loss of Par-4 in the context of PTEN haploinsufficiency leads to invasive PCa in mice.[20] Concomitant deficiency of both tumor suppressors has an impact not only on the progression of PIN lesions to invasive carcinoma, but also on tumor initiation, with a higher incidence of PIN lesions upon Par-4 loss. Interestingly, the combined mutation

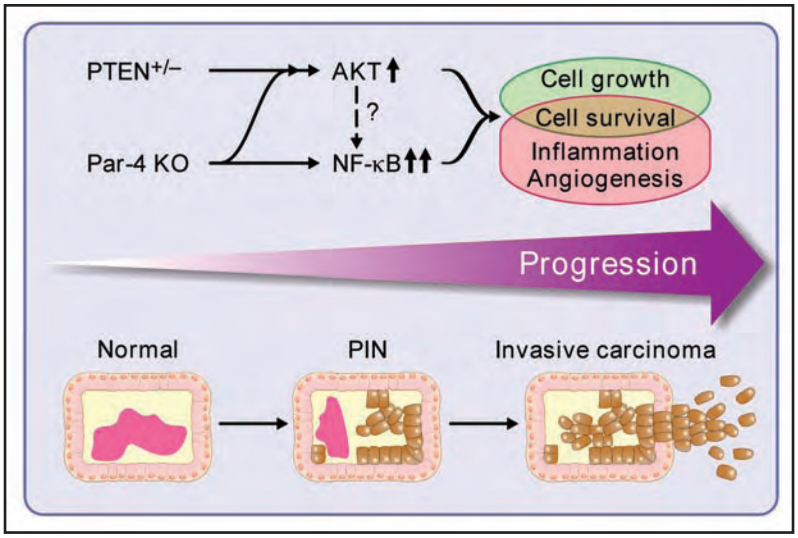

Figure 1. Cooperation of Par-4 deficiency and PTEN haploinsufficiency in prostate cancer progression. Par-4 loss cooperates with PTEN heterozygosity to promote invasive prostate carcinoma. The simultaneous inactivation of Par-4 and PTEN enhances Akt and leads to a synergistic stimulation of the NFκB pathway. This sets in motion complementary signals regulating cell growth, cell survival, inflammation and angiogenesis that collaborate in prostate cancer progression. It is not known whether Akt is able to directly impinge on the NFκB pathway in this system.

of Par-4 and PTEN regulates both proliferation and survival of prostatic epithelial cells, in contrast to the cooperation between PTEN and other tumor suppressors, which only affect proliferation. This is a unique feature of Par-4 and PTEN interplay that could be explained by the synergy of the two mutations on activation of the NFκB cascade, an important pathway in cell survival. Of note, the inactivation of both tumor suppressors results in the synergistic stimulation of NFκB, not only in PIN lesions but also in preneoplastic prostates.[20] This suggests that the activation of NFκB in the preneoplastic glands of the compound mutants could be a causative mechanism to promote invasive PCa. Par-4 deficiency also leads to an increase in Akt activation, and this effect is enhanced in the context of PTEN heterozygosity. Thus, the concomitant loss of PTEN and Par-4, in addition to modulating the Akt pathway, impinges on the NFκB cascade, which could unleash signals complementary to those elicited by Akt (Fig. 1). In this regard, two important inflammatory targets of NFκB, the cytokine IL-6 and the chemokine IL-8, are increased in the Par-4/PTEN compound-mutant prostates.[20] This might mediate the recruitment of inflammatory cells and facilitate an angiogenic response that could collaborate with proliferative and survival signals in the progression to an invasive phenotype.

Interestingly, the synergy between the inactivation of Par-4 and PTEN observed in the double-mutant mouse model is consistent with a significant association of Par-4 and PTEN expression levels in human PCa. That is, Par-4 loss is associated with PTEN loss and correlates with high Gleason scores in human PCa. Par-4 inactivation is mostly associated with aberrant de novo methylation of the *Par-4* promoter.[20] Of note, there is also an inverse correlation between Par-4 and PTEN levels and activation of the NFκB pathway, measured as p65 nuclear translocation and IL-6 levels, indicating that, in fact, activation of this pathway might account for the collaboration between these two tumor suppressors.

Par-4 and PTEN Interplay as a Safeguard Checkpoint in Tumor Progression

The combined loss of tumor suppressors is a hallmark of advanced human PCa and suggests a "one-by-one" hit model for tumor development in which there is a sequential loss of tumor suppressor genes. Research suggests that there is a line of defense against tumorigenesis composed of a number of tumor suppressors, each with the ability to control one or more cellular process through the specific pathways on which they act. This suggests that a network exists through which the different signaling and molecular events are integrated and coordinated to fine-tune cancer progression. To add to the complexity, tumor suppressor genes are also subject to countless regulatory mechanisms that ultimately control their activity, protein levels and function.

The fact that Par-4 KO prostates display a hyperplastic phenotype and do not progress to later stages in PCa, except in the context of PTEN haploinsufficiency, suggests that PTEN could act as a safeguard mechanism in the absence of Par-4 to prevent cancer progression. Thus, it is possible that PTEN levels could increase as a consequence of Par-4 deficiency, dampening the tumorigenic signaling cascades unleashed by the loss of Par-4. Figure 2 shows that this is actually the case, in that Par-4 KO prostates have increased PTEN protein and mRNA levels. These observations are consistent with the notion that PTEN acts as a checkpoint that limits hyperplastic proliferation and malignant transformation. That is, upon the loss of one tumor suppressor, the cell sets in motion compensatory mechanisms that induce other tumor suppressors to restrain tumorigenesis. Interestingly, such interplay between Par-4 and PTEN is also evident in PTEN heterozygous animals. That is, as shown in Figure 3, Par-4 levels are increased in PTEN$^{+/-}$ prostates, both at the protein and mRNA levels. These results suggest that the mutual regulatory interplay between Par-4 and PTEN expression may represent an additional safeguard mechanism in the transition from preneoplastic lesions to invasive cancer. This could also explain the cooperation of the two tumor suppressors, as the dual loss would result in the inactivation of this molecular brake. Invasive cancer, where checkpoint loss may have already occurred, would then be associated with reduced levels of both Par-4 and PTEN, a prediction that was confirmed by analysis of human prostate tumors.[20]

A similar scenario has previously been described in which PTEN is linked with other tumor suppressors, such as p53, in a complex relationship. PTEN has been reported to be a downstream target of p53 in mediating apoptosis,[39] and also to act upstream of p53 to regulate its expression levels and activity.[40,41] The physical binding of p53 and PTEN gives further support to their functional crosstalk.[42,43] Furthermore, total deletion of PTEN results in enhanced expression of p53 as well as p21, a direct

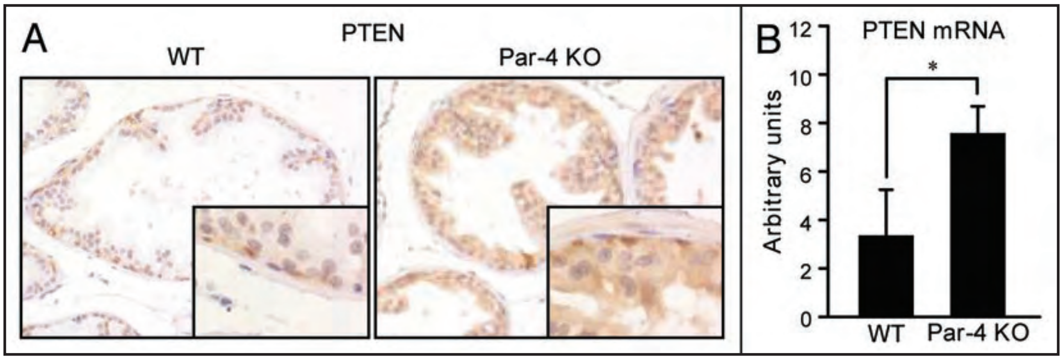

Figure 2. Increased levels of PTEN in Par-4 KO prostates. (A) Immunostaining showing increased PTEN levels in prostate epithelial cells in Par-4 KO mice. (B) PTEN mRNA levels are also induced upon Par-4 deficiency, as measured by QRT-PCR. n = 4 mice per genotype. $^{*}p < 0.02$.

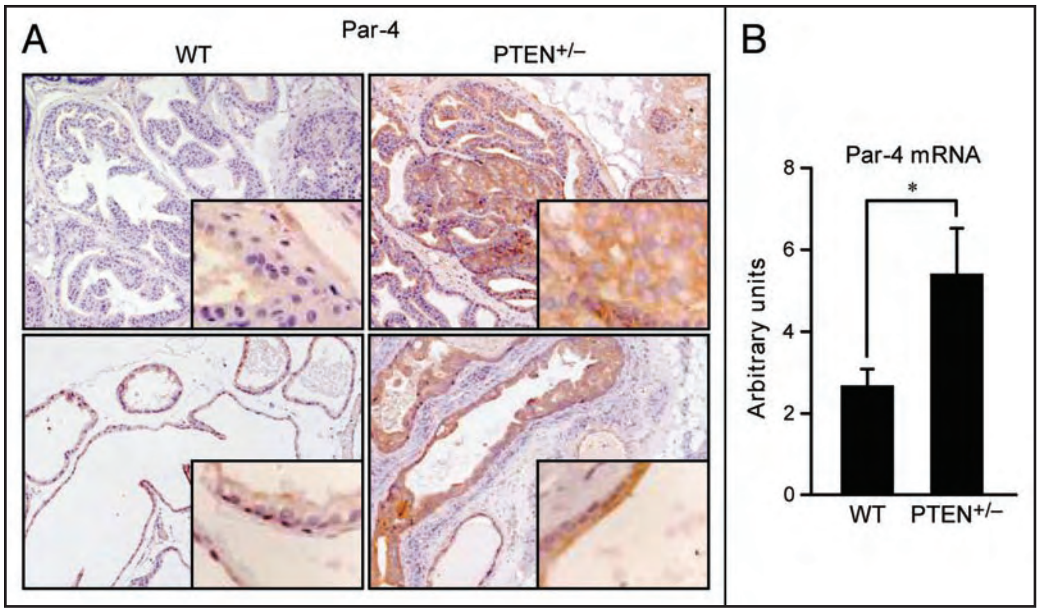

Figure 3. Induction of Par-4 in $PTEN^{+/-}$ prostates. (A) Immunostaining showing increased Par-4 levels in prostate epithelial cells of the anterior (upper) and dorsal (lower) prostate lobes in $PTEN^{+/-}$ mice. (B) Induction of Par-4 mRNA levels in dorsal prostates of $PTEN^{+/-}$ mice, as measured by QRT-PCR. n = 4 mice per genotype. $^{*}p < 0.01$.

transcription target of p53.[44] Consistent with this, combined inactivation of p53 and PTEN cooperates and accelerates tumor development.[44] Importantly, acute PTEN inactivation triggers p53-dependent cellular senescence as a checkpoint to restrain tumorigenesis.[44] Cellular senescence is commonly seen in early or precursor stages of cancer.[45,46] Interestingly, p27-mediated senescence has been also described in AKT1 transgenic and PTEN homozygous mice, where it is associated with the PIN phenotype. Consistent with this, loss of p27 in the context of AKT1 transgenic mice leads to increased proliferation, loss of senescence, and progression of PIN lesions to invasive PCa.[47] This crosstalk between PTEN and p53 or p27 resembles that of PTEN and Par-4, although whether or not the PTEN-Par-4 interplay triggers senescence is not known. The connections between Par-4 and other players in the PTEN tumor suppressor network and the mutual regulatory mechanisms controlling their levels, location and activity is still an open question that deserves further investigation.

Concluding Remarks

Par-4 is a novel tumor suppressor that is well positioned to be an integrator of the currently growing PTEN network. New studies are unveiling the cooperation of tumor suppressors to increase the threshold of a shared signaling event, or to set in place new molecular mechanisms. In this regard, Par-4 deficiency cooperates with PTEN haploinsufficiency to promote invasive PCa, and their simultaneous inactivation (in addition to enhancing Akt activation) sets in motion a unique mechanism involving the synergistic stimulation of NFκB. These observations provide further support for the increasingly recognized role of NFκB in cancer. Akt and NFκB pathways are both deregulated during prostate tumorigenesis, and their activation could offer complementary advantage to cells to further progress towards an invasive phenotype. These findings also suggest that the concurrent interruption of complementary signaling pathways could provide a new and effective avenue for cancer therapy. Specifically, combinatorial therapies targeting PI3K/Akt and NFκB signaling pathways may be an effective treatment for PCa.

In summary, recent evidences are unveiling that tumor suppressors exist in a finely tune and regulated network that integrates signals to coordinate molecular events that protect cells against tumorigenesis. Understanding the cross talks among each component of these cascades, and unraveling the intricate molecular

mechanisms that govern their connections will undoubtedly contribute to the development of new therapeutic strategies sorely needed for the treatment of aggressive forms of cancer.

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Review

Of the atypical PKCs, Par-4 and p62: recent understandings of the biology and pathology of a PB1-dominated complex

J Moscat*,[1], MT Diaz-Meco[1] and MW Wooten[2]

The recent identification of a novel protein–protein interaction module, termed PB1, in critical signaling molecules such as p62 (also known as sequestosome1), the atypical PKCs, and Par-6, has unveiled the existence of a new set of signaling complexes, which can be central to several biological processes from development to cancer. In this review, we will discuss the most recent advances on the role that the different components of these complexes have *in vivo* and that are relevant to human disease. In particular, we will review what we are learning from new data from knockout mice, and the indications from human mutations on the real role of these proteins in the physiology and biology of human diseases. The role that PKCζ, PKCλ/ι, and Par-4 have in lung and prostate cancer *in vivo* and in humans will be extensively covered in this article, as will the multifunctional role of p62 as a novel hub in cell signaling during cancer and inflammation, and the mechanistic details and controversial data published on its potential role in aggregate formation and signaling. All this published information is shedding new light on the proposed pathological implications of these PB1-regulators in disease and shows their important role in cell physiology.
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The protein kinase C (PKC) isozymes constitute a family of Ser/Thr kinases of the AGC group, which are subdivided into classical, novel, and atypical isoforms, based on structure and sequence homology and on their cofactor requirements. They all contain a C-terminal kinase domain linked through a variable 'V3' domain to an N-terminal regulatory domain, in which most of the structural differences reside (Figure 1a). The latter contains three functional elements: (i) an inhibitory region (pseudosubstrate), (ii) a C1 domain or zinc finger (one copy or tandem repeats), and (iii) a C2 domain. Distinct features of the regulatory domains contribute substantially to the particular roles of individual isoforms and to their respective mechanisms of action.

The atypical protein kinase C (aPKC) subfamily is composed of two members, PKCζ and PKCλ/ι. PKCλ is the mouse homolog of the human PKCι. The two aPKC isoforms are highly related, sharing an overall amino acid identity of 72%.[1] The conservation in their sequences is most striking in the catalytic domain, which is also conserved among other PKC isotypes that belong to the classical and novel subfamilies. In contrast, the regulatory domain of the aPKC subfamily diverts from other members of the PKC family; it has only one zinc finger, whereas the other PKCs have two[1] (Figure 1a). Through the zinc-finger domain, the aPKCs bind Par-4, a negative regulator of their enzymatic activity. Similar to the novel PKCs, the aPKCs lack the characteristic C2 domain that is present in the classical isoforms (Figure 1a). These important structural differences may explain why the aPKCs are insensitive to Ca^{2+}, diacylglycerol, and phorbol esters, which are potent activators of the other isoforms.[1]

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Abbreviations: APC, adenomatous polyposis coli gene; aPKC, atypical protein kinase C; BAG1/3, Bcl-2-associated athanogene-1/3; CHMP2B, charged multivesicular body protein 2B; CYLD, cylindromatosis gene; DKO, double knockout; DUB, deubiquitinating enzyme; ERK, extracellular responsive kinase; IHC, immunohistochemistry; IκB, inhibitor of κB; IKK, IκB kinase; IL, interleukin; IRAK, interleukin receptor-associated kinase; IRS, insulin receptor substrate; Jak1, Janus kinase-1; KO, knockout; LC3, microtubule-associated protein 1 light chain 3/MAP1LC3; MEF2, myocyte enhancer factor 2; MEK, MAPK/ERK kinase; MEKK, MEK kinase; MM, multiple myeloma; NBR1, next to BRCA1; NF-AT, nuclear factor of activated T cells; NF-κB, nuclear factor-B; NIK, NFκB-inducing kinase; NSCLC, non-small cell lung cancer OPCA-OPR-PC and AID; p62/SQSTM1, sequestosome-1; OVA, ovalbumin; Par-4, prostate androgen responsive-4; Par-6, partitioning defective-6; PB1, phox and Bem1p; PDB, Paget's disease of bone; PIK3CA, phosphoinositide-3-kinase catalytic alpha polypeptidePI3KR5, phosphoinositide-3-kinase regulatory subunit-5; PKC, protein kinase C; PTEN, tensin homolog deleted in chromosome ten; RANK-L, receptor activator of NF-κB-ligand; RHEB, Ras homolog enriched in brain; RPS6KB1, p70s6k ribosomal protein S6 kinase; SOD, superoxide dismutase; Th, T helper; TNF, tumor necrosis factor ; TRAF, TNF receptor-associated factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TrkA, tropomyosin-receptor- kinase; UBA, ubiquitin associated; XIAP, X-linked mammalian inhibitor of apoptosis protein

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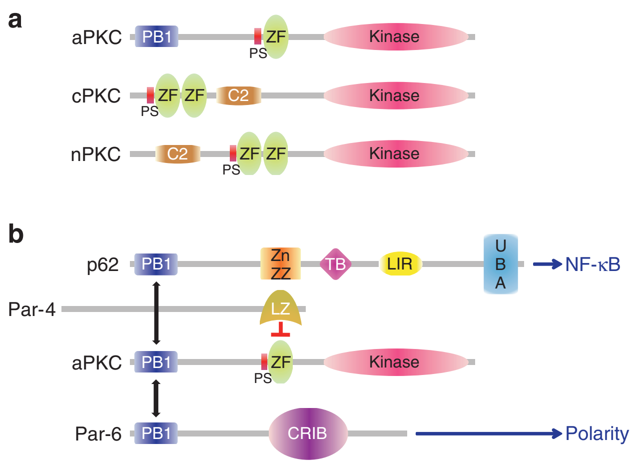

Figure 1 The atypical PKC network. (**a**) Domain organization of the different PKC subfamilies: aPKC, atypical PKC; cPKC, classical PKC; nPKC, novel PKC. PB1, PB1 dimerization domain; PS, pseudosubstrate region; ZF, zinc finger; C2, C2 domain. (**b**) The atypical PKC isoforms establish a network of protein interactions with adapter proteins (such as p62 and Par-6) binding through the PB1 domain and with regulators (such as Par-4) through their zinc finger domain. PB1–PB1 interactions confer specificity to the actions of the aPKCs. The interaction with p62 allocates the aPKCs in the NF-κB pathway, whereas through Par-6 the aPKCs regulate cell polarity. Domain architectures of the different proteins in the network are shown: Zn ZZ, ZZ-type zinc finger; TB, TRAF6 binding; LIR, LC3-interacting region; UBA, ubiquitin associated; LZ, leucine zipper, ZF, Zinc Finger; CRIB, Cdc42 Rac-interactive binding

The PB1 Domain: a Distinctive Characteristic of the aPKCs

The recent identification of the protein-interaction domain PB1, present at the N-terminus of the aPKCs, has opened new avenues to explore the roles of these kinase isoforms by looking for adapters and regulators that could shed light on their functions[2] (Figure 1). It is well known that PKCs are kinases that display little selectivity *in vitro* and when overexpressed in cells. This invokes the need for cellular mechanisms to confer functional specificity while preserving the capacity for cross talk, which is necessary for the regulation of complex biological processes. As the aPKCs have been implicated in diverse cellular functions, different adapter proteins must exist to achieve the required specificity during cell signaling.[3] In this regard, the PB1s are dimerization/oligomerization domains present in adapter and scaffold proteins, as well as in kinases, and serve to organize platforms to assemble the protein complexes necessary for such specificity. The PB1 domains are named after the prototypical domains found in Phox and Bem1p, which mediate polar–heterodimeric interactions. The PB1 domains comprise about 80 amino acid residues and are grouped into three types: type I (or type A), type II (or type B), and type I/II (or type AB). The type I domain group contains a conserved acidic DX(D/E)GD segment (called the OPCA motif) that interacts with a conserved lysine residue from a type II domain. Type I includes the PB1 domains of p40phox, MEK5, and NBR1, whereas type II occurs in p67phox, Par-6, MEKK2, and MEKK3. The type I/II PB1 domain, containing both the OPCA motif and the invariant lysine, is present in the aPKCs and in p62 (also known as sequestosome-1).[2,4] Type I and type II PB1 domains interact with each other in a front-to-back manner resulting in heterodimers in which acidic residues on the OPCA motif form salt bridges with basic residues of the type II PB1 domain.

Two-hybrid screenings in yeast identified p62 and Par-6 as selective adapters for the aPKCs.[3,5–8] Par-6 has been shown to be central to the control of cell polarity and, through its PB1 domain, allocates the aPKCs specifically in polarity-related functions[2] (Figure 1b). On the other hand, the p62/aPKC-signaling platform has a critical role in NF-κB activation[9] (Figure 1b). p62 interacts with PKCζ and PKCλ/ι, but not with any of the other closely related PKC family members. It is not a substrate, and does not seem to significantly affect the intrinsic kinase activity of PKCζ or PKCλ/ι. Moreover, it harbors a number of domains that support its role as a scaffold in cell signaling (Figure 1b). Thus, the formation of aPKC complexes with different adapters, scaffold proteins, and regulators, such as Par-6, p62, and Par-4, serves to confer specificity and plasticity to the actions of these kinases and to establish signaling networks that control several key cellular functions. However, the factors that determine which complex is formed at a given time and within a specific cell context remain to be identified (Figure 1). In this regard, new phosphorylation events have been identified that take place in the PB1 domain of PKCι, which may offer an explanation of a novel mechanism that could account for switches between the different interacting partners.[10]

Specificity and Function of the aPKCs

Before the recent availability of loss-of-function animal models, the similarity between PKCλ/ι and PKCζ and the lack of rigorously specific genetic and biochemical tools have hampered the effort to assign unique functions to the individual isoforms. For example, many studies have used commercially available antibodies that do not discern between the two aPKC isoforms. Also, attempts to inhibit aPKC enzymatic activity in cell cultures have made extensive use of a peptide with the sequence of the pseudosubstrate, which is identical for both aPKCs and therefore non-specific. In addition, the pseudosubstrate region is highly conserved among all the PKCs, not only the aPKCs, which casts serious doubts on studies that rely solely on the use of such reagents to establish the role of the aPKCs in a given function.[11,12] Moreover, the overexpression of dominant-negative and active forms of the two aPKCs does not necessarily discriminate between specific functions for each isoform, as these manipulations may impinge on pathways other than those with physiological relevance to each aPKC isotype. Despite these problems, however, the genetic inactivation of these isoforms *in vivo* by using knock out (KO) mouse technology is starting to shed light on their specific roles.

The fact that PKCλ/ι KO in mice is embryonic lethal at early stages, probably because of defects in cell polarity,[13] whereas the PKCζ KO mice are born in Mendelian ratios,[14] was a first indication of the different and specific functions that each of these kinases might have *in vivo*. The phenotypic analysis of PKCζ KO mice revealed a role for this kinase in the control of the immune response.[2] That is, PKCζ-deficient mice

displayed alterations in the development of secondary lymphoid organs, showing morphological defects in the spleen's marginal zone and Peyer's Patches, and a reduced percentage of mature B cells.[14,15] In keeping with this, the loss of PKCζ impaired B-cell survival and proliferation in response to activation through the B-cell receptor with no major alterations in T-cell proliferation.[15] In addition, the analysis of PKCζ-deficient mice unveiled an important role of PKCζ in the control of T-cell polarization programs, specifically during Th2 differentiation. In fact, PKCζ levels were increased during Th2 but not Th1 differentiation of CD4$^+$ T cells, and the loss of PKCζ impaired the secretion of Th2 cytokines *in vitro* and *in vivo*, Jak1 activation, and the nuclear translocation and tyrosine phosphorylation of Stat6, essential downstream targets of IL-4 signaling. Moreover, PKCζ KO mice displayed dramatic inhibition of ovalbumin (OVA)-induced allergic airway disease, strongly suggesting that PKCζ might be a good candidate for a novel therapeutic target in asthma, through the control of the IL-4 pathway.[16]

The aPKCs have also been implicated as important mediators in the control of cell survival through the activation of NF-κB in multiple cell systems.[3,17,18] Indeed, the genetic inactivation of PKCζ in mice supports a key role of this isoform in the activation of NF-κB in that PKCζ deficiency impairs NF-κB at two levels.[14] In the lung, where PKCζ is especially abundant, this kinase is required for the activation of IKK *in vivo*, whereas in other systems, such as embryo fibroblasts, endothelial cells, and B cells,[15,19] PKCζ controls the phosphorylation of the RelA subunit of the NF-κB complex at Ser311, enabling its interaction with the transcriptional co-activator CBP and subsequent gene expression.[20] Therefore, depending on the system, PKCζ could be considered an IKKβ kinase or may act downstream of IKKβ by controlling the transcriptional activity of the NF-κB complex.

On the other hand, unlike PKCζ, PKCλ/ι is required during development, as evidenced by the fact that PKCλ/ι-deficient mice die by embryonic day 9.5, likely because of abnormalities in development detected as early as day 6.5.[13] This phenotype is in agreement with that found for the disrupted expression of the aPKC orthologs in *C. elegans*,[21] *Xenopus*,[11] and *Drosophila*.[22–25] Functional knockouts in these organisms result in early embryonic lethality because of defects in polarity and asymmetric cell division.[21–25] The analysis of tissue-specific conditional PKCλ/ι-deficient mice is helping to elucidate the *in vivo* role of this atypical isoform. For example, the selective deficiency of PKCλ/ι in the liver resulted in increased insulin sensitivity,[26] whereas β-cell deficiency impaired glucose-induced insulin secretion and glucose tolerance.[27] Muscle-specific PKCλ/ι-deficient mice also provided evidence of its role in insulin action with a phenotype closely mimicking the human metabolic syndrome.[28] Whether PKCλ/ι is dysfunctional in acquired insulin resistance has yet to be determined in humans. In relation to its function in the immune response, PKCλ/ι, similar to PKCζ, has an essential role in Th2 establishment and allergic airway disease in mice.[29] Thus, the specific deletion of PKCλ/ι in activated T cells showed that this kinase is required for T-cell polarity and the activation of Th2-transcription factors, such as NF-κB, NFATc1, and GATA-3, linking defects in polarity to a functional impact on the Th2-mediated responses.[29]

The aPKC Regulators and Adapters Provide Clues to aPKC Function

Studying the *in vivo* function of the adapters and interacting partners of the aPKCs is an important tool to comprehensively address the physiological role of this kinase network. In fact, the analysis of the phenotype of Par-4-deficient mice confirmed the functional implication of the aPKCs in the immune response, mostly through their ability to regulate NF-κB. The available data support a model according to which the interaction of Par-4 with the zinc-finger region of the aPKC regulatory domain leads to the inhibition of aPKC enzymatic activity and the consequent reduction of NF-κB activity.[30] In this regard, the loss of Par-4 in embryo fibroblasts leads to increased aPKC and NF-κB activation.[31] Consistent with this, the NF-κB-dependent anti-apoptotic protein XIAP is expressed at significantly elevated levels in Par-4–null cells, which correlates with reduced caspase-3 activation and apoptosis.[31] In addition, Par-4 and PKCζ KO mice display opposite immune system phenotypes *in vivo*.[15,32] That is, although PKC$\zeta^{-/-}$ mice have impaired B-cell proliferation and function,[15] Par-4$^{-/-}$ mice have increased B-cell and T-cell proliferation.[32] Also, Par-4$^{-/-}$ T cells over-produce the Th2 cytokine IL-4,[33] whereas PKC$\zeta^{-/-}$ T cells show impaired Th2 polarization and IL-4 secretion *ex vivo* and *in vivo*.[16] As Par-4 binds and inhibits both aPKC isoforms, understanding the role of this negative regulator might help to predict the impact of inhibiting both isozymes *in vivo*, as could be the case with aPKC pharmacological inhibitors.

With regard to the adapter protein p62, it has been shown to be required for NF-κB signaling in several systems,[34–36] including *Drosophila*, in which a functionally relevant homolog termed Ref(2)P has been identified.[37] It is noted that p62 has been shown to be required for the sustained phase of NF-κB activation during T-cell differentiation, a process that is critical in asthma and other allergic diseases.[38] Interestingly, p62 levels are induced on T-cell differentiation,[38] suggesting that p62 is necessary to control biochemical events required for proper differentiation. The loss of p62 in T cells impairs their ability to produce Th2 cytokines *ex vivo* and is required for an optimal lung inflammatory response.[38] Therefore, p62, similar to PKCζ and PKCλ/ι, emerges as an important component of the signaling cascades regulating Th2 function and asthma.

On the other hand, Par-6 has been shown by genetic manipulations to be critically implicated in the control of cell polarity in *C. elegans* and *Drosophila*.[7,8] Although genetic data have yet to be produced to prove the role of the aPKCs and Par-6 in different aspects of mammalian cell polarity, overexpression analyses have implicated the Par-6/aPKC complex in the control of the epithelial–mesenchymal transition,[39] T-cell[40] and neuronal polarity,[41] and cell polarity in migrating astrocytes,[42] among other functions. Our recent demonstration of a role for PKCλ/ι in the control of T-cell polarity in knockout cells and mice is the first genetic demonstration of such a role for an aPKC in mammals. Therefore, the formation of aPKC complexes with different adapters and scaffold proteins serves to confer specificity and plasticity to the actions of these kinases.

The Cancer Biology of the aPKCs

Cell transfection and overexpression experiments suggested a role for the aPKC pathways in tumorigenesis and led to

different proposed mechanisms of action. However, the actual pathophysiological relevance of the different components of this network is now being established by studies in human cancer samples, and, importantly, in mouse genetic KO models as well.

In the case of PKCζ, a number of studies support its clinical relevance in tumorigenesis, including reports on altered expression in different types of cancer. Most of the studies, however, are restricted to microarray data with very limited reliable information on protein expression, as the antibodies used for these analyses recognize both aPKCs, thus precluding any conclusion regarding isoform specificity. It is noted that different studies in human tumor specimens have reported both the up- and downregulation of PKCζ expression, which may indicate tissue-specific roles for this aPKC isoform. That is, downregulated expression of PKCζ has been shown in glioblastoma,[43–46] lung cancer,[47–49] kidney renal clear cell carcinoma,[50] melanoma,[51] and pancreatic cancer,[52] whereas its upregulation has been shown in prostate cancer,[53,54] bladder cancer,[55,56] and lymphomas[57,58] All these studies point to an important role for PKCζ in human carcinogenesis. They have also unveiled a need for studies on the mechanism of PKCζ action to rigorously test whether it is an oncogene or, on the contrary, a tumor suppressor, and whether this depends on tissue-specific factors. A recent study from the Vogelstein laboratory, which could be of high relevance to this question, describes a genome-wide catalog of genetic changes in breast and colorectal tumors in which exons representing more than twenty-thousand transcripts from more than eighteen-thousand genes were analyzed.[59] In this study, they identified a few commonly mutated genes. Interestingly, their analysis led to the identification of a mutated form of PKCζ (S514F), along with mutations in other genes in human colorectal cancer that can be grouped bioinformatically in a pathway including, in addition to PKCζ, relevant signal transducers such as IRS2, IRS4, PIK3R5, PTEN, RHEB, RPS6KB1, and PIK3CA.[59] These results reinforce the earlier suggestion of a role for PKCζ in human tumorigenesis. Surprisingly, biochemical analysis of the consequence of the S514F mutation revealed that it significantly impaired PKCζ enzymatic activity.[49] This could be interpreted to mean that tumorigenesis is associated with impaired PKCζ and that, in fact, this kinase should be considered as a tumor suppressor. Consistent with this hypothesis, the overexpression of catalytically active PKCζ, but not the S514F mutant, in Ras-expressing NIH-3T3 fibroblasts restrained tumor growth in a xenograft *in vivo* model.[49] Furthermore, overexpression of the K281R kinase-dead mutant of PKCζ in the same *in vivo* model further enhanced tumorigenesis.[49]

Collectively, the results of these studies strongly suggest that PKCζ activity is important for restraining tumorigenesis *in vivo*. In keeping with this notion, PKCζ levels were low or undetectable in a significant proportion of human lung carcinomas.[49] A consideration of this finding, combined with the potential existence of inactivating mutations similar to the one identified in colon cancer patients, suggests that, in fact, the loss or inactivation of PKCζ will lead to tumorigenesis. A more physiological cancer model using PKCζ KO mice strongly supports these conclusions.[49] That is, the analysis of a mouse model of pulmonary adenocarcinoma in which oncogenic Ras is inducibly expressed in type II alveolar epithelial cells in response to doxycycline[60] showed that lung tumorigenesis is dramatically enhanced in a PKCζ KO background compared with that of WT mice.[49]

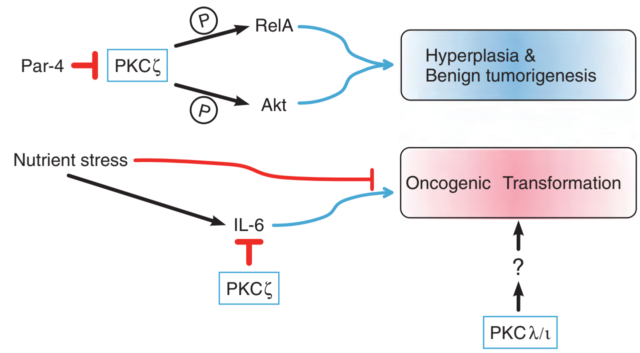

Figure 2 Dual role of PKCζ as a positive step in the regulation of Par-4-controlled hyperplasia (upper scheme), and in the regulation of nutrient stress during aggressive transformation (lower scheme)

A negative role for PKCζ in carcinogenesis contrasts with the apparent role of PKCλ/ι. That is, xenograft experiments in Ras-transformed embryo fibroblasts showed that the loss of PKCλ/ι dramatically impaired tumorigenesis (Figure 2). This is in agreement with results from Fields and co-workers showing that the loss of PKCλ/ι severely impaired intestinal tumor formation in the $Apc^{Min/+}$ mouse model.[61] It is quite remarkable that KO mice lacking PKCλ/ι show phenotypes completely opposite to PKCζ KO mice because the aPKCs share a striking degree of homology. Consistent with this, an increasing number of studies in humans show aberrant expression of PKCλ/ι in several cancer types,[62] but, contrary to PKCζ, PKCλ/ι is highly upregulated in all types of tumors, through gene amplification, increased mRNA expression, and protein overexpression. For example, PKCλ/ι protein levels are overexpressed in non-small cell lung adenocarcinomas (NSCLC), and the PKCλ/ι gene is frequently amplified in this tumor type.[63] Furthermore, these studies propose that PKCλ/ι expression is a useful marker of poor prognosis in this type of malignancy.[47,63,64] Moreover, PKCλ/ι is genomically amplified and overexpressed in ovarian cancers,[65–68] giving rise to the loss of apical–basal epithelial cell polarity.[66] A similar mechanism has been proposed in a recent report on overexpression and altered localization of PKCλ/ι in breast cancer, suggesting that the normal apicobasal polarity is lost on the progression of a breast lesion to invasive ductal carcinoma.[69] Gene locus amplification of PKCλ/ι has also been reported in esophageal squamous cell carcinoma.[70] PKCλ/ι is also upregulated in bladder cancer,[56] prostate cancer,[71] sarcoma,[72] lymphoma,[58] and multiple myeloma.[73] Taken together, these observations support a critical role for both aPKCs in cancer, but suggest that they are most likely playing opposite roles.

The Tumor Suppressor Role of Par-4

The fact that Par-4 binds both aPKCs, inhibiting their enzymatic activity, combined with the observation that its overexpression leads to increased apoptosis[31,74] is puzzling

in light of the opposing roles of the two aPKCs in carcinogenesis. The generation of Par-4 KO mice allowed the establishment of its actual *in vivo* role in cancer. It is noted that 80% of the Par-4 KO females presented endometrial hyperplasia by 9 months of age, and at least 36% developed endometrial adenocarcinomas after 1 year of age.[75] Par-4 KO males showed a high incidence of prostate hyperplasia and prostatic intraepithelial neoplasias.[76] These data strongly suggest that Par-4 could be a novel tumor suppressor in these two types of tumorigenic process. The analysis of human tumors supports this notion. That is, a study using cDNA arrays, quantitative reverse transcription-PCR, and immunohistochemistry detected Par-4 downregulation in approximately 40% of human endometrial carcinomas.[75] This study also showed that Par-4 promoter hypermethylation was detected in 32% of the tumors in association with low levels of Par-4 protein, and was more common in carcinomas positive for microsatellite instability,[75] indicating that promoter hypermethylation is the mechanism whereby Par-4 levels are downregulated in tumor cells.

Interestingly, recent studies show that Par-4 expression is lost in a high percentage of human prostate carcinomas (about 60%), with a significant association between Par-4 promoter methylation and lack of Par-4 expression, and a clear association with PTEN loss (Diaz-Meco MT and Moscat J, unpublished observations). This is very interesting because PTEN loss has been associated with, and shown to be critical in, prostate cancer.[77,78] Interestingly, Par-4 KO mice, similar to PTEN-heterozygous mice, develop only benign prostate lesions, but, importantly, concomitant Par-4 ablation and PTEN heterozygosity lead to invasive prostate carcinoma in mice (Diaz-Meco MT and Moscat J, unpublished observations). These results establish a cooperation between the two tumor suppressors, Par-4 and PTEN, as relevant for the development of prostate cancer in mice and possible in humans as well.

Of potential interest to establishing the generality of Par-4 as a tumor suppressor in cancer are data showing that the loss of Par-4 clearly enhances lung carcinogenesis in a mouse lung cancer model in which oncogenic Ras is introduced following a knock-in strategy, and inducibly expressed in an endogenous manner.[79] This and the doxycycline-inducible lung cancer model discussed above are physiologically relevant *in vivo* lung cancer models because the target cell, as in humans, is the type II pneumocyte that, in addition to the Clara cells, is the most likely precursor of human lung carcinoma.[60,80] Therefore, it appears that Par-4 is a tumor suppressor not only in the endometrium and prostate cancer but also in lung cancer. Consistent with this concept, a study of its expression in human NSCLC revealed that 47% of tumors were negative for Par-4 as determined by IHC, and that there was a clear correlation between the loss of Par-4 and tumor type. That is, 41% of the adenocarcinomas were negative for Par-4 expression, whereas only 6% of squamous cell carcinomas showed negative staining for Par-4. Also, when the adenocarcinomas were stratified by grade, it was clear that 74% of grade III tumors had lost Par-4 expression, whereas 59% of grade I-II tumors were negative for Par-4. Together, these data show that Par-4 is a relevant tumor suppressor gene in a significant number of human malignancies, strongly suggesting that this protein may have an important function in the prevention of, at least, endometrial, prostate, and lung cancer.

What Lies Downstream of Par-4?

As Par-4 manifests tumor suppressor activities[76,79] and is known to be involved in the binding and inhibition of PKCλ/ι and PKCζ,[74] it follows that both aPKCs are downstream targets of Par-4 in carcinogenesis. However, the fact that the inhibition or deletion of PKCζ enhances tumorigenesis,[49] similar to Par-4 deletion,[75,76,79] whereas the inactivation of PKCλ/ι blocks tumorigenesis (Figure 2), could be interpreted to mean that PKCλ/ι, but not PKCζ, is the bona fide downstream target of Par-4 in cancer. Surprisingly, the simultaneous genetic *in vivo* inactivation of Par-4 and PKCζ in double KO mice leads to the ablation of Par-4 deficiency-induced prostate hyperplasia and PIN (Diaz-Meco MT and Moscat J, unpublished observations). This observation is counterintuitive with regard to the data supporting roles for Par-4 and PKCζ as tumor suppressors. An explanation for this apparently paradoxical observation is based on the different roles of PKCζ in benign and malignant tumorigenesis, and is clearly linked to its mechanism of action. That is, it is possible that under conditions of hyperplasia in which tumors do not undergo metabolic stress, PKCζ, likely through its role as an activator of Akt[79] and NF-κB,[14] is necessary for the tumor proliferation and survival state unleashed by the loss of Par-4. That is the case for the development of benign hyperplasia produced in the Par-4 KO mice, which is completely eliminated by the simultaneous inactivation of PKCζ (Diaz-Meco MT and Moscat J, unpublished observations). However, in the case of more aggressive tumors, similar to those triggered by the expression of oncogenic Ras, which are characterized by a high degree of metabolic and nutrient stress, the loss of PKCζ would trigger another pathway that results in enhanced proliferation of the tumor cells[49] (Figure 2). The basis for this explanation was obtained in experiments where the growth of PKCζ-deficient Ras-transformed embryonic fibroblasts was investigated under conditions of plentiful nutrient availability, or under conditions of nutrient deficiency.[49] The latter situation is more likely to occur in the context of large, aggressive tumors. Interestingly, under conditions of nutrient and mitogen availability, the loss of PKCζ clearly impaired the proliferation of Ras-transformed cells, whereas under conditions of nutrient and mitogen deficiency the opposite phenotype was observed.[49] This indicates that PKCζ controls two antagonistic pathways for oncogenic cell proliferation (Figure 2): one that is required under normal conditions, and another that is inhibitory under nutrient-deficient stress situations.

The molecular mechanisms controlling either pathway need to be clarified, but it is interesting to note that the expression of Ras in lung tissues triggers the expression of a myriad of NF-κB-dependent genes in wild-type mice, which is completely inhibited in a PKCζ KO background.[49] Despite the inhibition of NF-κB, IL-6 levels are enhanced in Ras-transformed KO tissues and cells, which have been shown to be important in allowing these mutant cells to proliferate under highly aggressive tumorigenic and nutrient-stressed conditions.[49] The details of how PKCζ negatively influences IL-6 production

still need to be worked out, but are likely to involve epigenetic changes in the IL-6 promoter.[49]

The aPKCs, Mammalian Cell Polarity, and Cancer

The analysis of Par-4 KO and Par-4/PKCζ DKO mice revealed that PKCζ, in addition to having an important role in NF-κB gene expression, also regulates Akt by direct phosphorylation.[79] The ability of PKCζ to influence these two important signaling cascades is of great relevance for the mechanism of action of the Par-4/PKCζ cassette, at least in prostate and lung cancer.[79] However, the mechanism whereby PKCλ/ι regulates tumorigenesis *in vivo* is still a mystery. It is possible that PKCλ/ι could be having a major role in a signaling cascade different from those controlled by PKCζ, involving, for example, the Par-6/Par-3 polarity complex. Recent *in vitro* experiments link components of the polarity complexes to cancer, suggesting that the overexpression of Par-6 leads to increased growth factor-independent cell proliferation.[81] This, in turn, results in the hyperplastic development of polarized cells in three-dimensional acini, because of aPKC-dependent regulation of the MEK/ERK signaling cassette, but surprisingly without affecting cell polarity.[81] These results would be consistent with the already known ability of overexpressed aPKCs to modulate MEK[82,83] but are difficult to reconcile with the genetically well-established role of Par-6 in cell polarity, at least in lower organisms.[84] In the same vein, the manipulation of the levels of another polarity protein, named Scribble, also leads to changes in ERK activity, but in this case associated with alterations in the polarity phenotype.[85] Scribble is particularly interesting as its polarization has been shown to be under the control of PKCλ/ι in KO T cells,[29] and has also been shown in *in vitro* transfection experiments to channel apoptosis signals activated by the interaction of the oncogene ERB2 with the Par-6/aPKC module,[86] or by expression of the Myc oncogene *in vitro* in cell-culture model systems.[87] The interaction between Par-6 and PKCλ/ι in the human lung adenocarcinoma cell line A549 has also been suggested to be relevant for transformation, at least in overexpression and co-transfection experiments.[88] Therefore, a number of *in vitro* overexpression experiments implicate different polarity proteins in growth signaling control. However, *in vivo* demonstrations of the actual role of the aPKCs in cell polarity control in relevant cancer models are, unfortunately, still sorely lacking.

That the only aPKC existing in lower organisms has an important role in polarity is widely accepted.[2,84] In this regard, genetic studies in *Drosophila* and *C. elegans* support an important role for their aPKC, which is more closely related to PKCλ/ι than to PKCζ in cell polarity.[2,84] There is also a consensus that the loss of cell polarity could be relevant to carcinogenesis in mammalian cells.[84] Surprisingly, although the genetic inactivation of PKCλ/ι in mature T cells gives rise to polarity defects that translate into activation defects *in vitro* and *in vivo*,[29] PKCλ/ι deficiency in the liver or intestine, or PKCζ deficiency in the lung, does not result in detectable alterations in cell polarity under basal, non-transformed conditions.[26,49,89] It is believed that polarity loss should increase tumorigenesis and metastasis.[90] However, the only available *in vivo* data implicating PKCλ/ι in cancer shows that its inactivation prevents, rather than enhances, the tumorigenic phenotype of $Apc^{Min/+}$ mice.[61] This important observation goes against the purported role of PKCλ/ι in cell polarity and its impact on promoting transformation when ablated. It is possible that the simple KO of one of the aPKCs is not sufficient to drive the polarity phenotype in certain cell types and tissues, and that the simultaneous inactivation of PKCλ/ι and PKCζ is required for cell polarity effects to become detectable. However, this type of experiment, in which both aPKCs are deleted, could lead to confounding results, as the complete ablation of both aPKCs might lead to restructuring of different PB1 protein complexes, which could artifactually create novel signaling units whose physiological and real pathological significance would be questionable. Probably a better strategy would be to create genetic knock-in models with mutations in polarity genes, which could potentially be identified in unbiased genome-wide genetic screens of human tumor samples. The identification of the PKCζ S514F mutation described above[49,59] combined with the fact that its expression, at least in Ras-expressing xenografts, gives a phenotype consistent with that of the Ras-expressing PKCζ KO lung, might lead the way to genetic experiments that are more physiologically relevant to cancer.

The Role of p62 as a Hub in Cell Signaling

The fact that p62 is a molecule rich in different modular structures suggested, from the time of its initial identification, that it would have a critical role in the organization of cell signaling events that mediate a variety of cell functions[6,91] (Figure 3). Similar to the analysis of the aPKCs and Par-4, the analysis of mutations in humans and the phenotype of the KO mice have enlightened us with regard to its importance and mechanism of action, and have also brought about a new set of questions.

The first indication of a physiological function for p62 came from the discovery that it harbors several mutations associated with Paget's disease of the bone (PDB).[92,93] This suggested that a major role for p62 in humans was likely to be linked to the control of bone homeostasis.[94] In fact, p62 KO mice display defects in osteoclastogenesis *in vitro* as a consequence of inefficient activation of NF-κB in KO osteoclasts activated by RANK-L or TNFα, two critical cytokines in the activation of the osteoclastogenic response *in vivo*.[95]

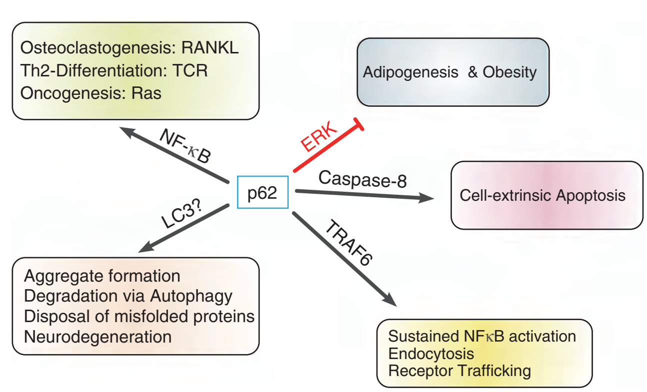

Figure 3 Different proposed roles for p62 in cell signaling and function

In addition, RANK-L triggers the formation of a p62–aPKC–TRAF6 complex in the pre-osteoclast cell line RAW 264.7 and in primary bone marrow-derived macrophages (BMDMs). However, given that the PKC$\zeta^{-/-}$ BMDMs, in contrast to BMDMs with PKCλ/ι knocked down, do not have osteoclastogenic defects, this suggests that PKCλ/ι may be the aPKC that acts in a signaling complex with p62 in this pathway[95] (Diaz-Meco MT and Moscat J, unpublished observations). Interestingly, the expression of p62 with a PDB mutation resulted in hyperactivated NF-κB and gain-of-function osteoclastogenesis, a phenotype consistent with that of the human disease.[96] Therefore, these studies, in combination with others showing a role for p62 in NF-κB activation at the level of the E3 ubiquitin ligase TRAF6 in other systems,[9,91,97] solidly established p62 as a critical player in the sustained activation of NF-κB. This link between p62 and NF-κB can also explain, at least in part, its role in cancer. Levels of p62 were dramatically increased in several tumor types, especially human lung cancers where more than 60% of lung adenocarcinomas and more than 90% of squamous cell carcinomas displayed elevated p62 protein levels, as determined by the immunohistochemical analysis of tissue microarrays.[9] Therefore, the facts that p62 is an activator of NF-κB, that this transcription factor has been shown to have a critical role in cancer,[98] and that human tumors have elevated p62 levels, strongly suggested that p62 could be an important player in tumorigenesis. In fact, experiments using the Ras-inducible lung cancer mouse model, discussed above, clearly showed that the p62 KOs show an outstanding resistance to carcinogenesis in this system,[9] very likely because of impaired Ras-induced NF-κB activation,[9] a critical pathway for Ras-induced survival.[99] In this regard, the genetic inactivation of PKCζ also inhibits Ras-induced NF-κB but, in contrast to p62, PKCζ KO mice display enhanced tumorigenesis.[49] This is mediated by an overproduction of IL-6 through a κB-independent mechanism.[49] Thus, although PKCζ and p62 both control the expression of NF-κB-dependent genes, suggesting that the p62-aPKC module is relevant for Ras-induced NF-κB, the mechanisms whereby they participate in the NF-κB pathway in response to Ras and the final outcome in carcinogenesis are different. That is, unlike PKCζ, p62 is required to activate the IKK complex through the activation of K63-mediated polyubiquitination of TRAF6 and to regulate the nuclear translocation of RelA/p65 in response to Ras.[9,35,95,97] PKCζ, on the other hand, can both positively regulate NF-κB at the transcriptional level and, at the same time, exert a negative effect on IL-6 production through a κB-independent pathway, which is responsible for the observed increase in carcinogenesis.[14,20,49] In this scenario, PKCζ could emerge as a critical step in the generation of inflammatory cytokines that might decide the final outcome of the carcinogenic process. The role of a p62–PKCλ/ι complex in Ras-induced carcinogenesis has not yet been addressed in *in vivo* mouse models.

In keeping with a role for p62 in cancer and the importance of NF-κB in this process, a recent study showed that knocking down p62 in stroma cells from multiple myeloma (MM) patients significantly inactivated the support of myeloma cell growth, likely because of the reduced production of IL-6, TNFα, and RANK-L by the p62-deficient stroma cells.[100] It is noted that aPKC activity was increased in marrow stromal cells from MM patients and that its activity was inhibited on knockdown of p62 in these cells.[100] This indicates that the p62–aPKC complex is an important step regulating NF-κB to modulate the MM stromal environment. This is particularly relevant in light of new information implicating NF-κB in multiple myeloma. That is, two laboratories, by using multiple-pronged genomic and gene-expression profiling approaches, have identified NF-κB-activating mutations in one-fifth of several hundred myeloma cell lines and patient samples.[101,102] These include gain-of-function mutations in positive regulators of NF-κB, such as NIK, NFκB1, NFκB2, and receptors of the TNF receptor superfamily, and loss-of-function mutations in genes encoding negative regulators such as TRAF3, TRAF2, and CylD.[101,102] Interestingly, there is also evidence that p62 interacts with CylD, which suggests a potential dual role of p62 in regulating not only the ubiquitination and subsequent activation of NF-κB signaling intermediaries but also its inactivation by deubiquitination through CylD.[103,104]

Consistent with this, the analysis of p62 KO mice also suggested a role for this molecule in controlling the accumulation of polyubiquitinated proteins, whose role in cell toxicity and tissue damage has been inferred from observations that they accumulate, along with p62, in several degenerative diseases.[105] For example, it has been shown that p62-deficient brains display increased accumulation of polyubiquitinated proteins, which can be because of the impaired proteasome activity[103] or decreased deubiquitination associated with p62 deficiency.[103] Interestingly, defects in autophagy also result in the accumulation of polyubiquitinated protein aggregates.[106,107] On the other hand, *in vivo* data in genetically modified mice support a role for autophagy in the control of p62 levels. That is, mice deficient in one of the critical autophagy genes, Atg7, display dramatically increased levels of p62 and an accumulation of polyubiquitinated aggregates that colocalize with p62.[108] Surprisingly, Atg7/p62 double KO hepatocytes, but not neurons, lack these aggregates, suggesting that p62 could, under some circumstances, have a still-to-be clarified role in their formation, at least in the liver.[108] These results have been interpreted to mean that p62, and possibly another scaffold protein termed NBR1,[109] might have structural roles in the formation of these aggregates. The precise role of these aggregates in cell pathophysiology still needs to be clarified. However, an elegant work recently published by Rubinsztein and coworkers[110] adds another angle to this problem that complicates the proposed role of p62 as a structural element in the formation of polyubiquitinated aggregates.[108,111,112] This group showed that the increased accumulation of p62 in autophagy-deficient cells led to the inhibition of the proteasome, which caused the accumulation of polyubiquitinated aggregates.[110] Obviously, in the absence of p62, the proteasome is not inhibited and the accumulated polyubiquitinated proteins are efficiently degraded in autophagy-impaired cells,[110] potentially explaining the lack of aggregates in the Atg7/p62 double KO mice without the need to invoke a hypothetical role for p62 in the building up of the aggregates.[108] This proposed hyperactivation of the proteasome in Atg7/p62-deficient livers would be in agreement with the

observation that the amount of polyubiquitinated proteins accumulated in that organ in these doubly mutant mice, as determined by SDS-PAGE, is lower than that in the single Atg7-deficient mice.[108]

One possibility is that p62, by binding the autophagy adapter LC3 and ubiquitinated proteins, brings aggregated or misfolded proteins to the autophagosome for their degradation.[113–115] Recent RNA interference and overexpression experiments in several cell culture systems suggest that possibility.[113–115] If this were the case, it would be very interesting because it would mean that cells have evolved a p62-dependent mechanism to dispose of excess proteins in the absence of a functional proteasome. However, it still needs to be clarified whether this putative p62-mediated autophagy mechanism is functional under physiological conditions, whether it is specific to pathological situations in which the proteasome is inhibited, or whether it is just an artifact of cell culture conditions. Again, this is complicated because of the connection of p62 to the proteasome, wherein p62 has been shown to have a critical role in the delivery of K63-polyubiquitinated proteins for degradation through a p62–S5a interaction.[116]

It appears that the proteasome cannot degrade insoluble misfolded proteins, which suggests that cellular surveillance pathways must exist to modulate protein quality control independent of the proteasome. There is the potential for p62 to be involved in such a mechanism; however, solid genetic evidence for this still needs to be produced. In this regard, recent findings reveal that the co-chaperones BAG1 and BAG3 are both involved in regulating the proteasomal and autophagic pathways,[117] where the BAG3/BAG1 ratio maintains appropriate protein homeostasis. Clearly then, the state of protein aggregation, which is a critical determinant of its solubility, and the degree of polyubiquitination are key regulatory factors that enable the sorting and clearance of protein aggregates. Thus, as the cell ages and experiences a decline in proteasome activity, it might have to rely increasingly on autophagy. In this scenario, the levels of p62 would confer a protective role by reducing the toxicity of these misfolded proteins. *In vitro* studies reveal that K63-linked ubiquitin may be preserved as a signal to selectively facilitate the clearance of tau and SOD-1 inclusions by autophagy.[118] With the development of antibodies selective for K63 modification, recent studies have revealed that the accumulation of inclusions that are immunopositive for K63-ubiquitin chains characterizes a genetic mouse model of 26S proteasome depletion, as well as human diseases involving proteasome function deficiencies, such as Alzheimer's, Parkinson's, and Huntington's disease.[119] The p62-mediated recruitment of misfolded proteins to autophagosomes could also involve the cooperation of other signals such as acetylation,[120] although further studies will be needed to define the exact means whereby this occurs. In situations such as cancer where p62 is upregulated,[9,121–123] cells could continue to survive and escape death thanks to the ability of p62 to activate NF-κB and to dispose of toxic aggregates, whereas, in a neurodegenerative disease such as Alzheimer's, where p62 levels are downregulated,[124] cells would die because of the accumulation of misfolded toxic proteins and a lower level of pro-survival NF-κB. Therefore, p62 could be having a decisive role in a hypothetical decision step that determines whether polyubiquitinated proteins reach a critical threshold to form aggregates or whether they are degraded through the autophagy or proteasome routes. The importance of this point of intersection *in vivo*, as well as its relationship to health or disease, needs to be clarified with further genetic studies in physiologically relevant organismal systems. In any case, all the literature surrounding the 'aggregate question' needs urgent clarification. That is, we need to understand which are the 'good aggregates,' and which are the 'toxic assets.' For example, the recent observation that Atg7 deficiency leads to hepatotoxicity, which is reduced in the double Atg7/p62 KO mice, is at odds with the idea that p62 is necessary to package polyubiquitinated aggregates to make them harmless, as the double-mutant livers do not develop the aggregates that are observed in Atg7-deficient mice.[108] In this regard, recent attempts to classify and understand the nature of these aggregates in yeast constitute one of the first serious efforts to better understand their actual roles and modes of action.[125] This, along with the clarification of the role played by the proteasome and DUBs in these processes, is absolutely necessary if we are to firmly establish a role for p62 in aggregate homeostasis.

Another potential function for p62 was suggested by several studies that position p62 as a key component in receptor turnover. This idea links back to the first report of p62 localization to the endosome–lysosome pathway.[126] That is, p62, through its UBA domain, associates with polyubiquitinated receptors, such as TrkA, and remains co-associated with the stage of late endosomes, although the receptor itself is degraded by lysosomes.[126] Early in the sorting pathway for TrkA, the receptor can be deubiquitinated by CylD through recruitment to the p62 scaffold,[127] resulting in receptor recycling. On the other hand, if a K63-polyubiquitinated receptor escapes this checkpoint, the chains are removed by proteasomal DUBs that associate with multivesicular bodies.[126] Recent observations further substantiate the finding that proteasomal DUBs are capable of degrading K63 chains.[128,129] In keeping with these data, mice deficient in p62 hyperaccumulate K63 chains and TrkA itself.[103] In this trafficking network, p62 remains co-associated and is required for presentation of the K63-polyubiquitinated receptor to the proteasomal DUBs. Thus, p62 appears to be necessary for the trafficking of cargo through the endosomal–lysosomal network. Interestingly, mutation in CHMP2B, a protein involved in endosome sorting, results in the accumulation of ubiquitinated proteins and p62, similar to what is seen in human disease.[130] Together, these findings, along with recent data from the Tooze laboratory,[131] lend further support to the temporal co-association of p62 with cargo early in the endosomal–lysosomal trafficking pathway and could therefore position p62 at the crossroads during the formation of autophagosomes in certain pathophysiological situations.

Taken together, all these published observations suggest that, indeed, p62 could have a more generalized role in trafficking of a wide array of substrates targeted for autophagy or lysosomal degradation. Although autophagy was once thought to be a relatively nonselective process, it is possible that p62 could provide selectivity through capture of specific substrates. Interestingly, p62 has recently been implicated in

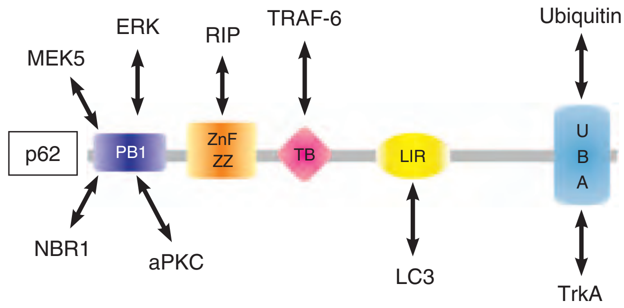

Figure 4 Interacting partners of p62

the degradation of peroxisomes[132] and has been observed in the midbody along with the ubiquitin-related protein Atg8 during abscission, suggesting that autophagy may be coupled to cytokinesis.[133] Given the conserved nature of the p62 function in flies and humans,[112] as well as the diversity in the physiological processes connected to the function of p62, it is clear that this protein serves as a rheostat to fine-tune multiple pathways that impinge on survival or death. If confirmed, p62's role would redefine autophagy as a selective process. The task at hand will be to identify, in physiologically relevant models, specific substrates that interact with p62 to be degraded by autophagy and the regulatory mechanisms that contribute to those interactions. In this regard, the other modules in p62, and its partners, the aPKCs, NBR1, ERK, MEK5, TRAF6, and CylD, could also have roles in this newly proposed p62 function, although the genetic evidence available so far does not support their involvement in this process (Figure 4).

On the other hand, it is possible that, rather than having a role in autophagy, p62 signaling might be modulated by this cellular process, which, because of p62's role as a pro-survival protein, could serve to fine-tune the decision of the stressed cells to live or die. Thus, although it is clear that the proposed role for p62 in the formation of the autophagic vesicles[113] is not supported by the genetic *in vivo* evidence from the p62 KO mice,[108] nevertheless, autophagy could be a mechanism to regulate p62-mediated signaling, as has recently been proposed for the transcriptional regulator MEF2D.[134] In this regard, the interaction of p62 with the E3 ubiquitin ligase TRAF6, which serves to promote the K-63 polyubiquitination of important intermediaries, such as TRAF6 itself,[97] IKKγ,[38] or TrkA,[127] could be inactivated if p62 is degraded during autophagy, which could result in the shutting down of the survival signaling pathways regulated by p62 through NF-κB, accelerating autophagic cell death. This could be relevant under conditions in which apoptosis is partially inhibited, for example in certain tumors. In addition, hypoxia-induced autophagy has been shown to deplete cellular levels of p62,[135,136] which triggers ERK activation that might affect cell growth and survival during hypoxic conditions.[135] Interestingly, these studies are in good agreement with previous data showing the negative regulation of ERK by p62 and its role in obesity in p62-deficient mice.[137]

Consistent with the notion that p62 is a 'signaling organizer' rather than 'garbage disposal' are the very recent data from the Ashkenazi laboratory.[138] Interestingly, they show that p62 cytosolic speckles constitute receiving entities of a signaling complex made up of the TRAIL receptor and a ubiquitinated form of Caspase 8.[138] The interaction of the Caspase 8 group with the p62 complex is necessary for the generation of higher-order Caspase 8 entities, which is required for TRAIL-induced Caspase 3 activation and apoptosis.[138] These results are reminiscent of previously published observations showing the formation of aggregate complexes including p62, TRAF6, and the kinase-adapter IRAK, which is necessary for a full activation of NF-κB.[35] According to this model, p62's role would be to organize different signaling complexes involving ubiquitinated signaling-relevant proteins that will determine the decision of cells to survive or undergo apoptosis. The fact that p62 can be degraded by autophagy suggests a mechanism whereby the cell could terminate these cascades under different situations, which may have pathophysiological implications in, for example, cancer. Whether the other components of the p62 hub such as the aPKCs are also involved in these 'signaling aggregates,' need to be clarified with further experimentation and will be essential for potential therapeutic intervention that would target the p62 'signaling organizer' complex.

Concluding Remarks

The aPKCs, PKCζ and PKCλ/ι, assemble in different signaling complexes through their interactions with scaffold proteins and regulators such as Par-6, p62, and Par-4. This generates two kinds of complexes depending on the domains of the aPKCs involved. That is, the aPKCs interact through their PB1 domains to form complexes with Par-6 and p62, whereas the complex formed with Par-4 is mediated by the aPKC's zinc-finger domain. These interactions set in place a signaling network that serves to confer specificity and plasticity to the actions of these kinases in the control of different key cellular processes. Recent data from knockout mice deficient in different components of this network are helping to shed light on the roles of these signaling molecules in physiologically relevant processes, such as cancer initiation and progression. However, there are still many open questions that need to be addressed in the future. For example, what are the factors that determine which complex is formed at a given time and within a specific cell context and is there cross talk between the different complexes? Similarly, the *in vivo* roles of the different components of the complexes need to be addressed more systematically in the same cellular contexts to allow for a direct comparison of the different biological outcomes and phenotypes. Once the *in vivo* functions of each component have been identified, such as the recent advances in understanding the role of p62, the task at hand will be to discern the makeup of the relevant signaling complexes in each physiological process and the contributions of the different binding partners to these functions.

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Immunological Reviews

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The atypical PKCs in inflammation: NF-κB and beyond

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Summary: From the very early days of nuclear factor-κB (NF-κB) research, it was recognized that different protein kinase C (PKC) isoforms might be involved in the activation of NF-κB. Pharmacological tools and pseudosubstrate inhibitors suggested that these kinases play a role in this important inflammatory and survival pathway; however, it was the analysis of several genetic mouse knockout models that revealed the complexity and interrelations between the different components of the PB1 network in several cellular functions, including T-cell biology, bone homeostasis, inflammation associated with the metabolic syndrome, and cancer. These studies unveiled, for example, the critical role of PKCζ as a positive regulator of NF-κB through the regulation of RelA but also its inflammatory suppressor activities through the regulation of the interleukin-4 signaling cascade. This observation is of relevance in T cells, where p62, PKCζ, PKCλ/ι, and NBR1 establish a mesh of interactions that culminate in the regulation of T-cell effector responses through the modulation of T-cell polarity. Many questions remain to be answered, not just from the point of view of the implication for NF-κB activation but also with regard to the *in vivo* interplay between these pathways in pathophysiological processes like obesity and cancer.

Keywords: *PKC, NF-κB, p62, Par-4, carcinogenesis, inflammation*

Introduction

The protein kinase C (PKC) family of proteins is a group of serine/threonine kinases that encompasses around 2% of the human kinome and forms a part of the AGC kinases, along with protein kinase A (PKA) and protein kinase G (1). PKCs are highly conserved in eukaryotes with different species showing divergent complexity, ranging from one isoform in *Saccharomyces cerevisiae* to 12 in mammals (2). All of these isoforms share a highly conserved catalytic domain and a more divergent regulatory domain at the N-terminus. These relatively conserved domains are linked through more variable hinge regions. The regulatory domain contains different structural domains that influence the sensitivity of each PKC to different stimuli, as well as their mechanism of regulation and function. There are layers of complexity and variations from one isoform to the other, but the pioneering work of Nishizuka and others in the early 1980s (3, 4) provided the basic framework for understanding the activation and structural properties of this family of kinases.

The 12 mammalian PKCs can be subdivided into four distinct subgroups based on the different topology of their regulatory domains (*Fig.* 1): the conventional or classical (cPKCs), the novel (nPKCs), the atypical (aPKCs), and the PKN group. The cPKCs include PKCα, PKCβ, and PKCγ and have a conserved region 1 (C1) as a tandem repeat that is structurally a double zinc finger and a binding pocket for the PKC effector diacylglycerol (DAG) and phospholipids (5, 6). They also contain a C2 domain that makes this subfamily responsive to calcium (7). The nPKCs comprise PKCδ, PKCε, PKCη, and PKCθ. Similar to the cPKCs, the novel PKCs are activated by DAG and phospholipids, but they are calcium independent. The aPKCs include PKCζ and PKCι (also known as PKCλ in mice). They are insensitive to calcium and DAG, most likely due to the lack of a C2 domain and to the single zinc-finger structure of the C1 domain. This group also contains a distinct structural domain called Phox/Bem 1 (PB1) at the N-terminus that is specific for this subfamily and that links these two isoforms (ζ and ι) to a network of PB1-containing proteins (8, 9) (see below). The PKN subfamily members (PKN1, PKN2, and PKN3) possess three leucine-zipper-like heptapeptide repeat 1 domains at their regulatory region, which bind Rho-GTP and regulate phosphorylation by phosphoinositide-dependent protein kinase 1 (PDK1) (10).

Activation of PKCs

The mechanisms involved in PKC activation have been extensively studied (11, 12). The current model proposes that, when inactive, PKC is auto-inhibited by its pseudosubstrate (an isoform-specific sequence present in the regulatory domain), which blocks the substrate-binding pocket in the kinase domain (13). This inactive state is preceded by a priming process through a series of serine/threonine transphosphorylation and autophosphorylation events that are required for maturation and stabilization (11, 12). To achieve a competent state, the kinase domain has to be phosphorylated on three (cPKCs and nPKCs) or two (aPKCs and PKNs) Ser or Thr sites, which stabilize the active kinase conformation. This process seems to require two upstream kinases. One is PDK1 to phosphorylate the activation loop in the kinase domain (14), and the other is the mammalian target of rapamycin 2 complex (mTORC2), which regulates phosphorylation of the turn motif and hydrophobic sites (when present) in the C-terminal tails of these kinases (15, 16). In the case of aPKCs and PKNs, an acidic phosphomimetic Asp or Glu is present in the hydrophobic motif instead of a phosphorylatable Ser or Thr. This acidic residue seems to bind the PDK-1-interacting fragment (PIF) pocket of PDK1, bypassing the requirement for the hydrophobic site phosphorylation (17, 18). Other regulatory or scaffolding components probably exist to regulate access to the PIF.

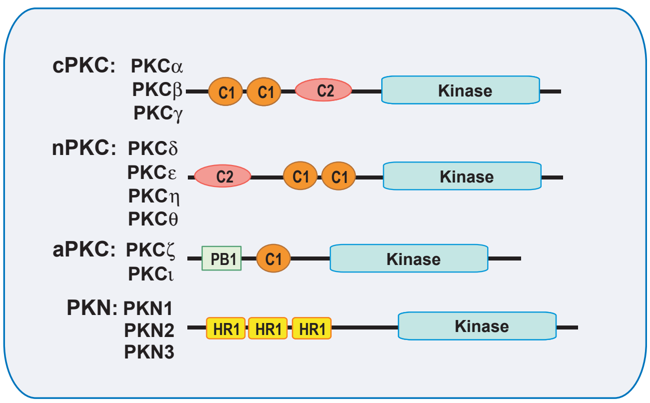

Fig. 1. Structure of the protein kinase C (PKC). Schematic representation of the different PKC subfamilies and their domain structural organization. The PKC family is divided into four structurally and functionally distinct subgroups according to their regulatory domains: the classical isoforms (cPKC), novel isoforms (nPKC), atypical isoforms (aPKC) and the PKC-related kinases (PKN). Conserved region 1 (C1) confers binding to diacylglycerol and phospholipids, and C2 senses calcium. PB1 (Phox/-Bem domain 1) is specific of aPKC and acts as a dimerization domain. Homology region 1 (HR1) confers small-GTPase binding properties to PKN.

The activation step takes place in response to the binding of lipid second messengers, allosteric effectors, or both, to specific domains at the regulatory region depending on the isotype. For those PKCs activated by DAG (cPKCs and nPKCs), increases in plasma-membrane DAG levels trigger the intracellular relocalization and activation. Sources of DAG include tyrosine-kinase receptor or G-protein-coupled receptor activation through the stimulation of phospholipase γ (PLCγ) or PLCβ. There are other less well-characterized mechanisms to generate DAG, such as the combined action of phospholipase D and phosphatidic acid hydrolase. PLCs produce the membrane lipid DAG and the soluble messenger inositol triphosphate upon cleavage of phosphoinositide 4,5-biphosphate. Increased DAG levels reversibly recruit cPKCs and nPKCs to the membrane through their zinc-finger (C1) domains (19). This membrane recruitment is generally considered as the primary event for cPKC and nPKC activation, although it does not fully explain the diverse intracellular localization of the different isoforms (20). Other protein modifications, such as tyrosine phosphorylation or proteolysis, might also be critical factors in mediating PKC activation (19).

The aPKCs, which cannot be activated by DAG, have been suggested to be sensitive to other lipids such as phosphatidylinositols (21), phosphatidic acid (22), arachidonic acid, and ceramide (23, 24). In addition, interaction with specific

binding partners could be an important mechanism to modulate activation and to confer spatial and temporal specificity to otherwise promiscuous kinases. For example, the C1 domain of the aPKCs that harbors a zinc-finger binds the protein Par-4, which blocks aPKC enzymatic activity (25). Par-4 is, therefore, a specific inhibitor of the aPKCs, most probably because its binding to the zing-finger competes with other stimuli. The other site for modulation by adapters is the PB1, but binding to this domain primarily affects localization and not the enzymatic activity of the aPKCs (26).

The PB1-domain network

The PB1 protein–protein interaction domain is unique to the aPKC subfamily of PKCs (*Fig.* 1). The identification of this domain has opened new avenues for exploring the specific functions of these kinases. Each adapter and regulator that has been found to interact with the PB1 domain sheds light on the physiological roles of the aPKCs. The PB1 domain is a modular scaffold domain, named after the prototypical domains found in Phox and Bem1p, which mediates polar heterodimeric interactions (8, 26). Besides the aPKCs, PB1s are found in adapter/scaffold proteins (such as p62, NBR1, and Par-6), and also in other kinases of the mitogen-activated protein kinase (MAPK) family, including MEK5α and MEKK3. This domain comprises about 80 amino acid residues and is conserved among animals, fungi, and plants. The human genome contains at least 13 PB1-containing proteins. Structurally, PB1 domains display the topology of a ubiquitin-like-β-grasp fold and are grouped into three types: type I (or type A), type II (or type B), and type I/II (or type AB) (9). The type I domain group contains a conserved acidic DX(D/E)GD segment (called the OPCA motif) that interacts with a conserved lysine residue of a type II domain. Type I includes the PB1 domains of p40phox, MEK5, and NBR1, whereas type II occurs in p67phox, Par-6, MEKK2, and MEKK3. The type I/II PB1 domain, containing both the OPCA motif and the invariant lysine, is present in the aPKCs, p62, and TFG (8). Heterodimeric assembly occurs between type I and type II PB1 domains and is considered to be a cellular mechanism for imposing spatial and temporal specificity during signaling (*Fig.* 2). The dimerization involves specific electrostatic interactions between the conserved acidic region of the OPCA motif from a type I domain with the conserved Lys residue from a type II domain. In addition, type I/II domains can homodimerize, at least theoretically. Indeed, p62 forms homo-oligomers, although such self-association has not been

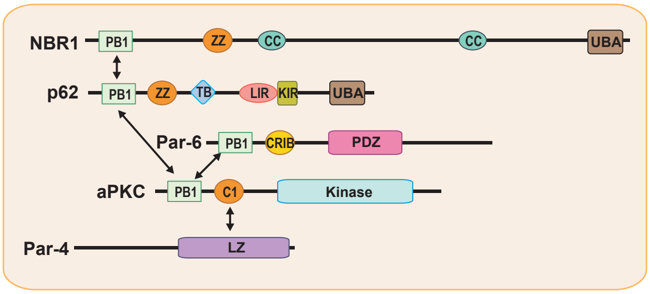

***Fig.* 2. The atypical protein kinase C (aPKC) signaling platform.** Schematic showing domain organization and network signaling mediated by aPKC and their adapters and regulators. The aPKCs interact with the PB1-containing adapters p62 and Par-6 to regulate specific functions. The scaffold p62 binds NBR1, a highly structurally related molecule with similar domain organization. Par-4 is a regulator and inhibitor of the aPKCs through binding to their C1 domain. C1, conserved region 1; PB1, Phox/Bem domain 1; LZ, leucine zipper; CRIB, Cdc42/Rac interactive binding; PDZ, PSD-95/Dlg/ZO-1; ZZ, ZZ-type zinc finger; TB, TRAF6-binding; LIR, LC3-interacting region; KIR, Keap-interacting region; UBA, ubiquitin-associated; CC, coiled coil.

described for the aPKCs or other proteins with this type of PB1 (27, 28).

p62 and Par-6 are selective adapters for the aPKCs (29–32). Par-6 has been shown to be central to the control of cell polarity and, through its PB1 domain, allocates the aPKCs specifically in polarity-related functions. The p62/aPKC signaling platform plays a critical role in NF-κB activation (33). p62 interacts with PKCζ and PKCι, but not with any of the other closely related PKC family members. It is not a substrate and does not seem to significantly affect the intrinsic kinase activity of the aPKCs (32). A p62 ortholog has been identified in *Caenorhabditis elegans* (T12G3.1) and in *Drosophila* [Ref(2)P], with both of these containing a conserved PB1 domain (34, 35). Moreover, p62 harbors a number of domains that support its role as a scaffold in aPKC signaling (33). Thus, the formation of aPKC complexes with different adapters, scaffold proteins, and regulators, such as Par-6, p62, and Par-4, serves to confer specificity and plasticity to the actions of these kinases and to establish a signaling network (36). However, the factors that determine which complex is formed at a given time remain to be identified.

Personal and historical narrative

Early studies on NF-κB activation by aPKCs

The initial studies on the role of the aPKCs in NF-κB signaling were done in our laboratory back in the 1990s and used *Xenopus laevis* oocytes as the model system (37). We designed the first peptides against the pseudosubstrate sequence to selectively block the activity of the different PKC isoforms (38).

This strategy was later broadly used incorporating myristoylated forms of the peptides to achieve cell permeability. Until that time, most of the experimental approaches to studying the PKCs involved their purported downregulation by chronic treatment with phorbol esters. However, it later became apparent that this strategy does not affect the aPKCs, because they do not bind phorbol esters. By using microinjection of specific inhibitor peptides into oocytes, we showed that an aPKC was required for insulin/Ras-induced NF-κB activation in *X. laevis* (37). Subsequent transfection experiments using kinase-defective dominant-negative mutants, overexpression experiments, or anti-sense approaches further supported a role for the aPKCs in the control of NF-κB activation (39–44).

These early studies were intended to establish the differential role of this subfamily of PKCs as a new pathway linked to NF-κB, and efforts were aimed at understanding the molecular mechanisms underlying the effect of these isotypes on the NF-κB cascade, as well as the level at which the effect was manifested. Initial reports suggested that PKCζ was upstream of the inhibitor of NF-κB (IκB) kinase (IKK) and able to bind the IKKβ to modulate its activation (45, 46). Later results using gene-deficient mice confirmed these initial observations while at the same time revealing a more complex and, most probably, tissue-specific role. Thus, PKCζ is required for IKK activation in the lung in response to tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), or lipopolysaccharide (LPS), but not in fibroblasts, in which its main function is to regulate NF-κB transcriptional activity at the level of RelA phosphorylation (47, 48).

Transcriptional control of NF-κB by PKCζ: Ser311

NF-κB regulates the expression of thousands of genes; thus, mechanisms need to be in place to fine-tune a process that is initially controlled by an all-or-nothing nuclear translocation pathway (49, 50). Results from several laboratories including ours (48, 51, 52) show that phosphorylations of the RelA subunit of the NF-κB complex fulfill that purpose. Under basal conditions, dimers of p50 NF-κB subunits are bound to gene regulatory elements in the chromatin. This prevents undesired uncontrolled activity by recruiting histone deacetylase, which keeps the expression of κB-dependent genes inhibited in the absence of stimuli due to the deacetylation of histones (53). According to the model put forward by Ghosh and co-workers (51, 53, 54), RelA is phosphorylated by PKA and/or MAPK and stress-activated protein kinase at Ser276 once p50-RelA heterodimers are released from IκB. This promotes the interaction of RelA with the transcriptional coactivator CBP (53). This interaction results in increased CBP-mediated histone acetylation, which in turn results in the generation of an 'open' permissive chromatin structure that allows the full transcriptional activity of the NF-κB complex (53). In addition to RelA serine 276 phosphorylation, serine 311 is also phosphorylated in response to TNFα (48). This serine residue is specifically targeted by PKCζ, which has been shown, through genetic manipulations, to be required for full NF-κB transcriptional activity *in vivo* and in cell culture experiments (55). Interestingly, both phosphorylation residues reside in the Rel homology dimerization domain and are required for the recruitment of CBP (48, 51).

Phosphorylation of serine 311 occurs in a region proximal to the site where other post-translational modifications take place to modulate the strength and duration of NF-κB nuclear activity (56, 57). Among these modifications, acetylation of Lys310 is one the best characterized (49). It is required for the full transcriptional potential of NF-κB and is important for modulating NF-κB-dependent inflammatory response (49, 58). In addition, Lys 314 and 315 are methylated by SET9 to terminate the NF-κB signal (59, 60). Both acetylation and methylation have been shown to have a functional interplay with phosphorylation to fine-tune transcriptional activation. Thus, for example, acetylation of Lys310 is blocked in the absence of Ser276 phosphorylation, as this phosphorylation event is required to recruit CBP/p300 and allow acetylation at Lys 310 (61). In addition, acetylation at this residue impairs methylation of Lys 314 and 315, which are important events for the ubiquitination and degradation of chromatin-associated RelA (57). Recent results show another layer of control via crosstalk between phosphorylation of serine 311 and monomethylation at lysine 310 (62). An unbiased screening of human protein lysine methyltransferases led to the discovery of SET domain-containing 6 (SETD6) as the methyltransferase responsible for the monomethylation of chromatin-associated RelA at lysine 310 (62). Importantly, the methylated form of RelA resides in a histone H3-rich region near the promoters of several NF-κB genes, which suggests that methylated RelA represses gene expression under basal conditions (63). Consistent with this hypothesis, the inactivation of SETD6 leads to increased κB-dependent transcription under basal conditions and, importantly, under stimulated conditions as well (62). These observations indicated that the lysine 310-serine 311 sequence could be a 'hotspot' for the transcriptional regulation of NF-κB by chromatin acetylation–methylation. Methylated lysine 310 binds a protein termed GLP, which along with its partner G9a, promotes the methylation of H3 at lysine 9 in chromatin regions with

repressed transcription (64). Therefore, lysine 310 methylation of RelA by SETD6 under basal conditions results in the recruitment of GLP, which methylates histone H3 and consequently keeps chromatin in a 'closed' state incompatible with active transcription (62). When cells are incubated with TNF, GLP is released; the chromatin is 'opened' and efficient activation of κB-dependent gene transcription takes place (Fig. 3). The role of PKCζ in this model is critical because its activation in TNF-treated cells leads to the phosphorylation of serine 311, which displaces GLP from RelA allowing the demethylation of chromatin and the ensuing enhanced transcription of κB genes (62). A recent study in which the SETD6-RelA peptide complex structure was determined suggests a structural basis for the methyl-phospho switch between Lys310 and Ser311 to regulate the localized chromatin state and gene expression (65). In summary, the PKCζ-mediated phosphorylation of serine 311 promotes the opening of chromatin by increasing its acetylation, mediated by CBP recruitment, and inhibiting its methylation through the release of GLP from methylated lysine 310. These are important observations, but the physiological significance of lysine 310 methylation and serine 311 phosphorylation *in vivo* still needs to be determined at an organismal level by analyzing knockin mice with point mutations in those sites. In addition, more mechanistic details are necessary for a detailed understanding of the precise molecular mechanism whereby phosphorylated serine 276 and serine 311 cooperate to recruit CBP, as well as how the phosphorylation of serine 311 controls the functional interaction of SETD6 and GLP with RelA. In any case, a role for this new pathway in inflammation has been demonstrated in genetically PKCζ-deficient cells, which are incapable of an adequate inflammatory response to TNF and IL-1. Moreover, this pathway also appears to play a role in cancer, as a reduction in SETD6 in transformed cells led to increased tumorigenic potential *in vitro* and *in vivo* (62). The possible correlation of SETD6 or GLP levels with tumor patient survival as well as the existence of potential mutations in lysine 310 or the hyperphosphorylation of serine 311 in patient tumor samples needs to be determined to establish the relevance of these novel modifications to human cancer.

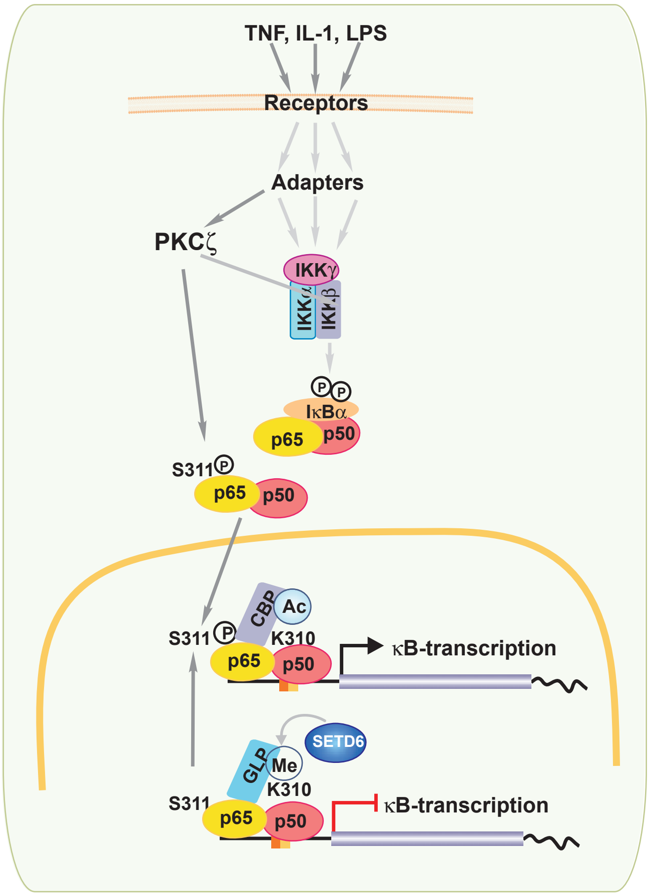

Fig. 3. Role of protein kinase C (PKCζ) in nuclear factor-κB (NF-κB) activation. The binding of different ligands to their respective receptors in the plasma membrane triggers the recruitment of specific adapters for each receptor that orchestrate the formation of a signalosome complex that includes two catalytic (IKKα and IKKβ) and one regulatory subunit (IKKγ). This complex phosphorylates IκB, which is subsequently ubiquitinated and degraded through the proteasome system, releasing NF-κB (the more classical components of which are p65-p50 heterodimers), which is free now to translocate to the nucleus and interact with elements in the promoter of inflammatory and survival genes harboring κB-elements in their promoters. PKCζ phosphorylates p65 at Serine 311 (S311), an important residue for recruiting the CBP coactivator complex. This event promotes acetylation of Lysine 310 (K310) to activate transcription. Under basal conditions, p65 is methylated (Me) at K310 by SETD6, promoting recruitment of GLP, which leads to the repression of κB-dependent transcription. Upon ligand binding, phosphorylation of S311 by PKCζ blocks methylation to activate the system. Depending on tissue specificity PKCζ could also act as an IKK kinase.

The aPKC pathway in the control of NF-κB in *Drosophila*

The NF-κB pathway is remarkably conserved in *Drosophila* and is critical for the control of the innate immune response (66). The RelA homologs in *Drosophila*, dorsal-related immunity factor (Dif) and dorsal, are essential for the synthesis of the antimicrobial peptide drosomycin in response to the activation of the Toll pathway by fungal pathogens (67, 68). Both dorsal and Dif are retained in the cytosol by the IκB homologue cactus, whose phosphorylation and subsequent degradation release these transcription factors allowing their translocation to the nucleus (67, 68). Parallel to the Toll pathway, there is another pathway in *Drosophila* that involves the kinase dTAK1, which serves to control the degradation of relish (68). Relish is the fly homolog of NF-κB1/NF-κB2 and is required for the synthesis of immune response proteins, namely anti-microbial

peptides, including diptericin (67). Interestingly, knocking down the *Drosophila* aPKC (DaPKC) ortholog with RNA interference in *Schneider* cells inhibits drosomycin expression but not that of diptericin, indicating that DaPKC is located specifically in the Toll anti-fungal pathway (34). DaPKC knockdown does not affect cactus or relish degradation but does inhibit drosomycin transcriptional activity (34). Furthermore, DaPKC phosphorylates Dif, the fly homolog of RelA, which suggest a conserved role for the aPKCs in the regulation of NF-κB transcriptional activity (34). In this regard, the p62 ortholog, Ref(2)P binds not only DaPKC but also the fly homologue of TRAF6 (dTRAF2). Overexpression of Ref(2)P is sufficient to activate drosomycin, and its depletion severely impairs Toll signaling, which is more evidence for the conservation of the aPKC pathway and the importance of this kinase in the regulation of NF-κB and the innate immune response (34, 69).

Proof of concept and new pathways unveiled in knockout mice

The phenotypic analysis of PKCζ-deficient mice confirmed the role of PKCζ in the control of NF-κB *in vivo* in the immune system, specifically in B cells (55). The first indication of such a role came from the analysis of PKCζ total knockout (KO) mice. These mice displayed alterations in the development of secondary lymphoid organs, showing morphological defects in the spleen's marginal zone and Peyer's patches, and a reduced percentage of mature B cells (55, 70). Interestingly, this correlated with deficient B-cell survival and proliferation in response to B-cell receptor (BCR) activation but not to the stimulation of other receptors (70). *In vivo*, PKCζ deficiency resulted in an impaired adaptive response with significant decreases in the production of immunoglobulin G1 (IgG1), IgG2a, IgG2b, and IgA. With regard to the phenotype of PKCζ-deficient T cells (see below), there were also deficiencies in IgE (70). Biochemically, it was found that expression of at least three κB-dependent genes was impaired in PKCζ-deficient B cells in response to BCR activation, with little or no changes in the nuclear translocation of NF-κB (70). This observation is consistent with the notion that PKCζ does not control IKK activation, except in the lung where PKCζ is abundantly expressed, or in overexpression experiments. Instead, it controls the regulation of NF-κB transcriptional activity by phosphorylation of serine 311 (48, 55).

The role of PKCζ in B-cell proliferation and NF-κB activation contrasts with the lack of effect of PKCζ deficiency on T-cell proliferation (70). However, our data showed that PKCζ KO does have a measurable and reproducible effect on T-cell differentiation towards the T-helper 2 (Th2) lineage (71). We found that the loss of PKCζ impaired the *in vitro* polarization to Th2 without effecting Th0 or Th1 differentiation (71). Interestingly, we also observed defects in the activation of GATA3, a hallmark or Th2 differentiation (72). The nuclear translocation of RelA was also impaired in PKCζ-deficient cells (71). However, as the activation of other transcription factors such as c-Maf and signal transducer and activator of transcription 6 (Stat6) was also impaired, this strongly suggested that the role of PKCζ was not restricted to NF-κB activation during Th2 differentiation but, rather, that PKCζ was playing a more fundamental role in this process. Consistent with this idea, we found that PKCζ was important for IL-4 signaling, which, along with signals emanating from the T-cell receptor (TCR), is essential for the activation of the Th2 differentiation program (71). We showed that Stat6 phosphorylation in response to IL-4 stimulation was impaired even in mature, undifferentiated, PKCζ-deficient T cells as compared with their wildtype (WT) controls (71). The precise mechanism has not been totally clarified, but it has been shown to involve the recruitment of PKCζ to the activated IL-4 signaling complex and the direct phosphorylation of Janus kinase 1 (Jak1) by PKCζ, which modulates Jak1 activation to phosphorylate Stat6 (73). These observations have important repercussions *in vivo*. For example, PKCζ deficiency impaired the ovalbumin-induced allergic airway inflammation in mice, a typical *in vivo* Th2 response (71). Adoptive transfer experiments demonstrated that this effect was not due to PKCζ deficiency in the stroma but, instead, to a genuine autonomous T-cell effect (71). Collectively, these observations unveiled a previously unanticipated role for PKCζ in a pathway separate from the NF-κB pathway. The fact that PKCζ is also important in IL-4 signaling indicates that it is a versatile kinase that influences processes in addition to the inflammatory response.

The novel role for PKCζ in IL-4 signaling is also important in T-cell-mediated fulminant hepatitis, another physiologically relevant *in vivo* response. The discovery of the role for PKCζ in this pathological situation came from analyzing the effects of PKCζ deficiency in mice injected with concanavalin A (ConA), a well-established model of fulminant hepatitis (74). Previous studies have suggested that the activation of NF-κB was a suppressor of liver apoptosis in fulminant hepatitis (75, 76). However, in contrast to expectations, even though PKCζ-deficient mice showed impaired NF-κB activation in the liver, which should have resulted in impaired survival, they showed reduced damage to the liver and a healthier state than their WT controls (73). This finding indicated that even though PKCζ is required for NF-κB activation, it must play an

additional role in a pathway required for T-cell-induced hepatitis. Interestingly, we showed that the loss of PKCζ inhibited the induction of serum IL-5 and liver eotaxin, two important mediators of liver damage (73). As eotaxin is synthesized by hepatocytes and liver sinusoidal endothelial cells, whereas IL-5 is produced by natural killer T (NKT) cells, these results imply that the loss of PKCζ affects the function of both liver and NKT cells. The adoptive transfer of PKCζ-deficient liver mononuclear cells (including a large proportion of NKT cells) into PKCζ KO mice was unable to restore ConA-induced hepatitis, whereas the adoptive transfer of WT mononuclear cells into PKCζ-deficient mice did restore liver damage (73). This outcome is similar to the *Stat6*$^{-/-}$ mouse phenotype (77), thus supporting the idea that PKCζ is a physiologically relevant regulator of Stat6. We also observed more liver damage in PKCζ-deficient mice under these conditions, as compared with WT mice, due to the inhibition of NF-κB, which deprived the liver of its protecting signals (78). Taken together, these findings reinforce the notion that PKCζ plays a physiologically relevant role as a dual regulator of NF-κB and Stat6.

Mice in which the negative regulator of aPKC, Par-4, had been knocked out revealed a phenotype biochemically consistent with the aPKCs being responsible for an important step in NF-κB activation. Embryo fibroblasts from Par-4-deficient mice displayed increased NF-κB activation and decreased stimulation of C-Jun N-terminal kinase (JNK) (25, 79). Interestingly, when the immunological phenotype of these mutant mice was analyzed, it was clear that Par-4 deficiency resulted in an increased proliferative response of peripheral T cells when challenged through the TCR, accompanied by enhanced cell cycle entry and inhibition of apoptosis, with augmented IL-2 secretion (80). From a biochemical point of view, the TCR-triggered activation of NF-κB was increased, resulting in a corresponding increase in IL-4 production. These results are in good agreement with Par-4 inhibiting the ability of PKCζ to modulate the IL-4 signaling pathway and Th2 differentiation, and with a role for the aPKCs in NF-κB activation and mature T-cell proliferation (71, 80). However, they contrast with our observations that the loss of PKCζ does not affect mature T-cell proliferation or NF-κB activation (70). These paradoxical findings could be explained by the fact that Par-4, in addition to targeting PKCζ in T cells, would also inhibit PKCλ/ι, the latter being responsible for the enhanced proliferative effects of Par-4-deficient mature T cells.

To address this important question, we analyzed the phenotype of mice in which PKCλ/ι was selectively ablated in activated T cells. For this study, we crossed PKCλ/ι$^{fl/fl}$ mice with CreOX40 mice in which the expression of Cre was under the control of the *Tnfrsf4* locus (81). OX-40 is expressed almost exclusively in activated T cells, especially CD4^{+} cells, only upon stimulation (81). This strategy resulted in a mutant mouse line in which PKCλ/ι was expressed at normal levels in immature thymocytes and naive T cells and, as predicted, was deleted only upon T-cell activation, thus avoiding embryonic lethality and preventing potential confounding effects resulting from the deletion of PKCλ/ι during development or in resting cells (82). Surprisingly, these mice did not show a proliferative defect, but like PKCζ-deficient mice, they had impaired Th2 differentiation *in vitro* and *in vivo* in the disease model of ovalbumin-induced allergic airway inflammation (82). However, and again surprisingly, the mechanism whereby PKCλ/ι impinged on the Th2 differentiation program was quite different from that of PKCζ. PKCλ/ι, in contrast to PKCζ, was not required for IL-4 activation of Stat6. Rather, its genetic ablation led to a global shutdown in the activation of a myriad of Th2-relevant transcription factors such as nuclear factor for activated T cells (NFAT), NF-κB, and Stat6, in addition to the master regulatory gene in Th2 differentiation, GATA3 (82). These results indicated that PKCλ/ι, whose levels, like those of PKCζ, are increased during Th2 differentiation, affects some fundamental T-cell function that, when impaired, results in a global inhibition of transcriptional signaling.

One fundamental T-cell function that could be affected by the loss of PKCλ/ι is T-cell polarity, a mechanism essential for T-cell activation and in which the aPKCs have been genetically implicated in lower organisms (83, 84). Of great functional relevance in this regard, we showed that the ability of the polarity marker Scribble to localize to one of the poles of the activated T cell was severely impaired in PKCλ/ι-deficient T cells (82). Also, the asymmetric polarization of CD44 relative to CD3 was diminished in the mutant T cells, as was the proper localization of Crtam, another recently discovered marker of late polarization (82). Collectively, these results indicate that PKCλ/ι deficiency in activated T cells leads to impaired polarity during late T-cell activation, which results in a general shutdown of the Th2 transcriptional machinery. Therefore, although PKCλ/ι is a direct upstream IKK kinase *in vitro* (46) and PKCζ is necessary for the activation of NF-κB transcriptional activity by phosphorylation of serine 311 *in vitro* and *in vivo* (48, 55), the role of PKCλ/ι *in vivo*, at least in T cells, is more complex and related to the role in regulating cell polarity for the aPKCs that was suggested in lower organisms. This role seems to be restricted to PKCλ/ι since the loss of PKCζ had no effect on T-cell polarity, although it was essential for IL-4 signaling towards Stat6 in Th2 differentiation (71, 82).

PKCζ, an anti-inflammatory signaling molecule in adipocytes

The fact that PKCζ plays a critical role in IL-4 signaling is relevant to asthma as well as other pathological situations. Recently, we found that PKCζ is an important anti-inflammatory molecule in obesity-induced inflammation and the ensuing insulin resistance and type 2 diabetes (85). These findings are based on studies highlighting the importance of adipose tissue inflammation in the induction of glucose intolerance and insulin resistance during obesity (86–90). Interestingly, the genetic inactivation of peroxisome proliferator-activated receptor γ (PPARγ) or PPARδ in macrophages prevented the alternative activation of macrophages (type 2), which resulted in a tonic type 1 hyperinflammatory state, and the ensuing glucose intolerance and insulin resistance during obesity (91, 92). There are, however, other scenarios, such as in JNK-deficient mice, in which inflammation is orchestrated by the adipocytes, which control the inflammation-induced insulin resistance in the liver through the generation of IL-6 (88). It is likely, depending on the different signaling pathways, that the hematopoietic system, the stroma, or both are responsible for these processes. Our data showed that PKCζ KO mice exhibit a hyperinflammatory state during obesity that correlates with a glucose intolerance and insulin resistance. In addition, we showed that even though PKCζ is involved in the generation of M2 macrophages, PKCζ ablation in the non-hematopoietic compartment but not in the hematopoietic system was sufficient to drive inflammation and IL-6 synthesis in the adipose tissue, which, based on the phenotype of PKCζ/IL-6 double KO mice, accounts for insulin resistance during obesity (85). Therefore, PKCζ emerges as a positive regulator of NF-κB, and also as a as a critical negative modulator of IL-6 in the control of obesity-induced inflammation in adipocytes through its positive role in IL-4 signaling, which is also relevant in allergic responses and Th2 differentiation, as well as in T-cell mediated fulminant hepatitis.

aPKC adapters and NF-κB in T-cell activation and beyond

Our own work and that of others led to the identification of two PB1-containing scaffolds for the aPKCs: Par-6 and p62 (93). Par-6 has been implicated in the control of T-cell polarity in several systems (83). However, there is still no genetic *in vivo* evidence in mammals that it is actually relevant for T-cell polarity (83, 93). Our data showing that PKCλ/ι is, in fact, important in T-cell polarity suggest that Par-6 might also be functionally relevant to that function (82). Future studies should address this important function using conditional KO models such as that reported here for PKCλ/ι.

Because p62 binds both aPKCs, it is conceivable that it also plays a role in T-cell differentiation towards the Th2 lineage. Early studies from our laboratory using overexpression and anti-sense-mediated downregulation of p62 demonstrated that it is an important player in TNFα-mediated NF-κB activation owing to its ability to interact with the intermediary receptor interacting protein 1 (94). Of relevance for the *in vivo* phenotype of p62-deficient mice, we found that it binds TNF receptor associated factor 6 (TRAF6), the IL-1 and LPS intermediary in the NF-κB pathway (95). It should be borne in mind that p62 is a complex scaffold protein that has several structural modules that serve to engage diverse signaling pathways (33) (Fig. 4). However, its ability to interact with TRAF6 is especially relevant from a pathophysiological point of view, due to the role of TRAF6 and NF-κB in bone remodeling through the control of osteoclastogenesis, and the fact that a series of well-defined mutations in p62 are associated with the Paget's disease of bone (PDB), a genetic disorder characterized by aberrant osteoclastogenesis and bone homeostasis (96, 97). This is a pathway controlled by receptor activator of NF-κB ligand (RANK-L) that is essential for osteoclastogenesis (98). Of relevance for the role of p62 in NF-κB activation *in vivo*, we have shown that RANK-L triggers the formation of a p62-aPKC-TRAF6 complex in RAW 264.7 cells and primary bone marrow-derived macrophages (99). The *in vitro* and *in*

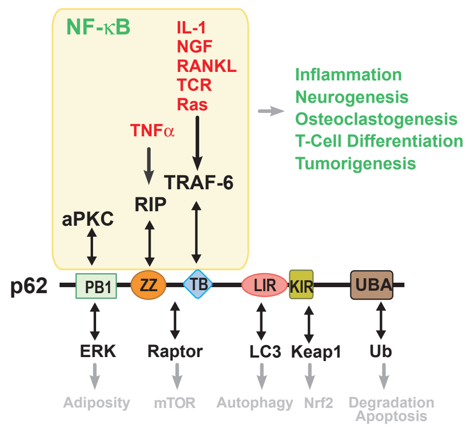

Fig. 4. p62 is a signaling hub. Schematic showing domain organization and network signaling mediated by p62. The signaling adapter p62 is a critical mediator of important cellular functions owing to its ability to establish interactions with various signaling intermediaries. p62 plays a key role in nuclear factor-κB (NF-κB) through specific binding to aPKC, RIP, and TRAF6 to control inflammation, neurogenesis, osteoclastogenesis, T-cell differentiation and tumorigenesis. PB1, PB1 dimerization domain; ZZ, ZZ-type zinc finger; TB, TRAF6-binding; LIR, LC3-interacting region; KIR, Keap-interacting region; UBA, ubiquitin-associated.

vivo expression of p62 with a PDB mutation resulted in hyperactivated NF-κB and gain-of-function osteoclastogenesis, which is in agreement with the phenotype of the human disease (99–101). Mechanistically, p62 promotes its own oligomerization and that of TRAF6 leading to enhanced E3 ubiquitin-ligase activity that is important for NF-κB activation (102, 103). Of special relevance for T-cell biology, p62 levels are induced in T-cell differentiation, and its genetic ablation in mice results in impaired ovalbumin-induced allergic airway inflammation *in vivo* and Th2 differentiation *ex vivo* (104). Therefore, p62, similar to PKCζ and PKCλ/ι, emerges as an important component of the signaling cascades regulating Th2 function and asthma (71, 82, 104).

The connection between p62 and NF-κB is also relevant in cancer (33). Levels of p62 are high in several human tumor types, especially in human lung cancers where more than 60% of lung adenocarcinomas and more than 90% of squamous cell carcinomas show elevated p62 protein levels (103). Consistent with a role for p62 in cancer through its ability to regulate the TRAF6-NF-κB axis, we showed that the loss of p62 in a Ras-inducible lung cancer mouse model resulted in resistance to carcinogenesis in this system, likely as a consequence of impaired Ras-induced TRAF6 and IKK activation and the ensuing stimulation of NF-κB (103). This led to increased ROS production by the p62-deficient cancer cell due to the lack of NF-κB-dependent ROS detoxifying enzymes, which resulted in enhanced apoptosis in Ras-expressing p62-deficient pneumocytes and fibroblasts (103). In addition to its role in lung tumorigenesis, p62 has also been shown to be involved in multiple myeloma (105). But in this case its actions are not in the tumor cell but in the stroma (105). That is, knocking down p62 in stromal cells from multiple myeloma patients abrogated the support of myeloma cell growth, as a consequence of reduced production of inflammatory cytokines such as IL-6, TNFα, and RANK-L, which correlated with the inhibition of aPKC activity in the stromal cells (105). This finding suggests an unexplored role for p62 and the aPKCs in the tumor microenvironment, which is particularly relevant in light of new information implicating members of the NF-κB cascade in multiple myeloma.

Several studies have demonstrated that p62 interacts with NBR1, another PB1-containing adapter that cannot interact with the aPKCs (106). The modular organization of NBR1 is quite similar to that of p62, which suggests that they might be involved in similar pathways or even perform redundant functions. To address this question, our laboratory has generated an NBR1$^{fl/fl}$ mouse line that we have used to test the potential role of this protein in Th2 differentiation. As in the case of PKCλ/ι, NBR1 was selectively deleted in activated T cells and their ability to differentiate to Th2 cells was determined. Interestingly, like T cells deficient in PKCλ/ι, PKCζ, or p62, the genetic inactivation of NBR1 also led to impaired Th2 differentiation *ex vivo* and *in vivo*, as assessed by a reduced response to ovalbumin-induced allergic airway inflammation *in vivo* (107). From a mechanistic point of view, the loss of NBR1 did not affect NF-κB activation but did inhibit GATA3 as well as NAFTc1 and Stat6 activation (107). The effects on Stat6 were secondary to reduced IL-4 levels in the mutant T cells (107). However, NBR1 actions on NFATc1, although not yet totally defined, seemed to be more direct (107). Intriguingly, as for PKCλ/ι, the loss of NBR1 resulted in defects in T-cell polarity, which in contrast to PKCλ/ι deficiency, did not lead to NF-κB inhibition (82, 107). Therefore, subtle variations in the polarity complex give rise to different transcriptional signaling alterations all resulting in impaired Th2 differentiation and reduced allergic responses *in vivo* (107). Collectively, these studies highlight a very interesting link between T-cell polarity and transcriptional control. A key question is how these complexes interact to provide this important layer of gene expression control so critical for T-cell differentiation. The first aspect of this mechanism is the recruitment of the different players to the immunological synapse (IS) as part of the polarity process. In this regard, the loss of NBR1 had no effect on the recruitment of PKCλ/ι to the IS (107). Likewise, the genetic inactivation of PKCλ/ι did not affect the IS translocation of NBR1 (107). This observation is consistent with a model whereby NBR1 and PKCλ/ι are mutually independent. However, the translocation of p62 to the IS was dependent on NBR1 but independent of PKCλ/ι (107). Surprisingly, the translocation of PKCλ/ι was independent of p62, but that of NBR1 was not (107). Together, these results show that the likely interaction between p62 and NBR1 is required for their translocation to the IS, whereas the translocation of PKCλ/ι is independent of both adapters, which, likewise, translocate independently of PKCλ/ι. The second aspect of this mechanism is polarity itself determined by the recruitment of polarity markers such as scribble and talin to the IS. In this regard, it is clear from our previous data that the lack of NBR1 or p62 during T-cell activation leads to a significant reduction in the recruitment of these two polarity markers to the IS, indicating that upon T-cell activation, NBR1 is normally translocated to the IS, independently of PKCλ/ι but in conjunction with p62. This could explain why NBR1 or p62 deficiency leads to impaired polarity during late T-cell activation. It also implies that they are likely anchored to different adapters in the IS. Consistently, detailed biochemical studies demonstrated the

interaction of PKCλ/ι with p62 as well as that of p62 with NBR1, but NBR1 was never shown to interact with PKCλ/ι (107). Therefore, although upon T-cell activation NBR1 and PKCλ/ι are able to interact with their common partner, they do not make direct contact with each other, even though both are located in the IS and both are critical for normal Th2 function. Although these observations establish for the first time the existence of a PB1 domain-mediated complex important for Th2 differentiation, there are still many unanswered questions with regard to how these complexes influence polarity and transcriptional activation through NF-κB or NFAT1c. It is expected that whereas the loss of p62 impairs NF-κB activation by the TCR, that of NBR1 does not, although both require each other to be recruited to the IS. Therefore, p62 must be in two different complexes binding either PKCλ/ι or NBR1 (107). At the IS, the two complexes would control different aspects of NFATc1 signaling. On the one hand, PKCλ/ι controls NFATc1 at the transcriptional level through the nuclear translocation of NF-κB (82), whereas NBR1 is likely to be responsible for the specific activation of NFATc1 in an NF-κB-independent manner (107).

Par-4, a negative regulator of NF-κB through the aPKCs

Our analysis of the phenotype of Par-4-deficient mice definitively tested the *in vivo* role of the aPKCs in NF-κB activation, at least in the immune response (80). The interaction of Par-4 with the zinc-finger domain of PKCζ, PKCλ/ι, or both resulted in repression of the enzymatic activity of both aPKCs that, in turn, provoked the inhibition of NF-κB function (25). Consequently, the loss of Par-4 in embryo fibroblasts from KO mice led to enhanced PKCζ and NF-κB activities, with functional repercussions on cell survival (79). However, possibly the most compelling evidence for the existence of a Par-4/aPKC cassette in the control of NF-κB *in vivo* came from the analysis of the immune response in mice deficient in Par-4 or doubly deficient in Par-4 and PKCζ, as compared to their WT counterparts and PKCζ single KO mice. First, we showed that Par-4 and PKCζ KO mice displayed opposite immunological phenotypes *in vivo* and *ex vivo* (70, 80). Whereas PKCζ-deficient mice were characterized by impaired B-cell proliferation and function (70) as well as impaired Th2 differentiation (71), Par-4-deficient mice had increased B-cell proliferation and their T cells overproduced the Th2 cytokine IL-4 *in vitro* and *ex vivo* (80).

Collectively these observations indicate that Par-4 is a physiologically relevant, naturally occurring negative regulator of inflammation through its ability to negatively affect the aPKC-NF-κB tandem. However, Par-4 was initially identified as a pro-apoptotic molecule in cell cultures (25, 108), and our *in vivo* mouse work has established that this is also true *in vivo*, specifically in prostate cancer (109). This is not totally unexpected as NF-κB is a prosurvival transcription factor and it is known that its ablation *in vitro* and *in vivo* gives rise to increased apoptosis, although its role in cancer seems to be organ or tissue specific (110). In this context, our data analyzing the tumor phenotype of Par-4-deficient mice adds another layer to the regulatory pathways controlling carcinogenesis through NF-κB and its crosstalk with other relevant signaling pathways. Interestingly, we found that upon aging, at least 80% of Par-4 KO females developed endometrial hyperplasia and at least 36% developed endometrial adenocarcinomas after 1 year of age (111). Also, Par-4 KO males had a high incidence of prostate hyperplasia and intraepithelial neoplasias (112), strongly suggesting that Par-4 is in fact a tumor suppressor. This was confirmed in human cancers in which Par-4 was found to be downregulated in 40% of human endometrial carcinomas (111), and it was lost in a 60% of human prostate carcinomas (109) and in 47% of non-small cell lung carcinomas (113). In this case, there is a clear correlation between the loss of Par-4 and tumor type, since 41% of the adenocarcinomas were negative for Par-4 expression whereas only 6% of squamous cell carcinomas showed negative staining for Par-4. Also, when the adenocarcinomas were stratified by grade, it was clear that 74% of grade III tumors had lost Par-4 expression, whereas 59% of grade I-II tumors were negative for Par-4 (113). Therefore, the role of Par-4 as a potential tumor suppressor linked to its ability to modulate cell survival through the aPKC-NF-κB cassette is likely relevant in human cancers. Recent *in vivo* results from our own laboratory in physiologically relevant mouse models confirmed this hypothesis and revealed the existence of unexpected signaling crosstalk orchestrated by the Par-4-aPKC module important for cancer and the associated inflammatory response. This evidence was obtained in two mouse cancer models relevant to the types of human tumors in which we have found inhibition of Par-4 expression. One is the PTEN-deficiency-driven prostate cancer model. In this model we found that Par-4 deficiency resulted in a phenotype very similar to that of PTEN-heterozygous mice, which developed only benign prostate lesions (109). However, the concomitant homozygous inactivation of Par-4 in a heterozygous PTEN background led to invasive prostate carcinoma in mice (109). These are very important observations because they establish a physiologically relevant cooperation between Par-4 and PTEN in the development of prostate cancer in mice and likely in humans.

Consistent with the data from human lung cancers, Par-4 not only inhibits PTEN-deficiency-driven carcinogenesis but also keeps the tumorigenic process at bay even when it is activated by an oncogenic signal. In this regard, we have also shown that the loss of Par-4 clearly enhanced lung carcinogenesis in a highly relevant mouse model of this disease (113). Since Par-4 is reduced primarily in adenocarcinomas and because this type of lung cancer is the one that best correlates with the expression of oncogenic Ras (114), we hypothesized that loss of Par-4 would promote tumorigenesis triggered by this oncogene and possibly others. This hypothesis was supported by crossing the Par-4 KO mice with a model of pulmonary adenocarcinoma in which oncogenic Ras was introduced by a knockin strategy and was inducibly expressed in an endogenous manner (115). Upon Ras expression, these mice develop lung adenomas and adenocarcinomas, with the likely target cell being the type II pneumocyte (115, 116). This is a physiologically relevant model for human cancer, as it has been reported that, in addition to Clara cells, type II pneumocytes are the most likely precursors of human lung adenocarcinomas (116–118). Interestingly, mice lacking Par-4 showed increased lung adenocarcinomas in this model, which was associated with enhanced cell proliferation *in vivo* as determined by increased Ki67 staining compared with WT lungs (113).

These findings demonstrate that Par-4 loss results in benign neoplasias and enhanced tumorigenesis in at least two mouse models driven by either the loss of a tumor suppressor or the induction of an oncogene. Also, we have shown that Par-4 is lost in human tumors. As Par-4 is a negative regulator of NF-κB through aPKC, the critical question is whether these novel effects account for enhanced NF-κB production in Par-4-deficient tumors. As the loss of Par-4 leads to synergistic cooperation with PTEN heterozygosity for the induction of prostate carcinomas, it could be predicted that they would also cooperate to activate NF-κB if this were the causative mechanism of the enhanced tumorigenicity of the double mutant prostates. Our laboratory demonstrated that this was indeed the case, as we found that whereas the single insufficiency of Par-4 or PTEN was enough to modestly activate NF-κB, this activation was synergistically enhanced in the double-mutant prostates, even at the preneoplastic stage, indicating that enhanced NF-κB activation was not a consequence but likely the cause of the cooperation between the two tumor suppressors (109). This concept was further supported by the analysis of a large array of human prostate tumor samples, reinforcing the physiological relevance of these findings (109). This cooperation was cell autonomous, as demonstrated in several mouse and human cell culture model systems in which it was also shown that the genetic or pharmacological inactivation of NF-κB dramatically reduced Par-4/PTEN deficiency-driven tumorigenicity (109). Mechanistically, PTEN is a negative regulator of Akt activation, and we found its activity to be enhanced in the PTEN mutant prostates (109). Surprisingly, we also found enhanced Akt activity in the Par-4 KO prostates. Importantly, activation of Akt was additive in the double Par-4/PTEN mutant prostates, not synergistic like that of NF-κB (109). These observations indicate that (i) Akt is a novel downstream target of Par-4 and (ii) Akt activation does not correlate with the synergistic induction of prostate adenocarcinomas. Previous observations also indicate that the transgenic expression of activated Akt in prostate is not sufficient to drive formation of invasive carcinomas (119). It is possible, as proposed by Baldwin's laboratory, that there was cross-talk between Akt and NF-κB in PTEN-deficient prostate cancer cells (120) and that this would be exacerbated in the context of Par-4 deficiency through the aPKCs. It was found that Par-4 can actually control Akt because this is a substrate of PKCζ (113). That is, PKCζ has been shown to phosphorylate Ser473 and Ser124 *in vivo* and *in vitro*, and these phosphorylations are antagonized by Par-4 (113). Ser473 is also targeted by the mTORC2 complex, and our experiments using rictor knockdown strategies demonstrated that PKCζ is not the major contributor to phosphorylation at this site but that it is for Ser124 (113, 121). The phosphorylation of this residue, along with that of Thr450, is important in facilitating the phosphorylation of Thr308 and Ser473 by PDK1 and mTORC2, respectively (121, 122). Therefore, the Par-4/PKCζ complex emerges as a critical cassette in the control of Akt and NF-κB in at least two types of neoplasias, prostate and lung. These observations also unveil the complexity of the aPKC's actions, which are not limited to the activation of NF-κB, but which also include the regulation of Akt the Jak1/Stat6 pathway.

Perspectives: conclusions and outstanding questions

It is clear from this review that the atypical PKCs regulate different mechanisms depending on the cell system and organ, likely due in part to the fact that they are relatively promiscuous kinases whose activities must be regulated by the interaction with adapters. Specifically, PKCζ has both pro-inflammatory and anti-inflammatory effects, which complicates the interpretation of the mouse KO phenotypes but, at the same time, underscores the complexity of the inflammatory process. PKCζ is also a tumor suppressor and future

studies should elucidate the contributions of its connection to NF-κB and/or Stat6 in carcinogenesis, especially from the point of view of the different cell types that populate the tumor microenvironment. Also, the connection between the aPKCs and p62 in inflammation and cancer should be addressed using genetic *in vivo* models, as should the link between novel PB1-containing adapters, such as NBR1, in metabolism and cancer.

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Cooperation between Par-4 and PTEN in Prostate Tumorigenesis

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Prostate cancer is one of the most common neoplasias in men. The disease is complex in its development and response to therapy. Therefore, a better understanding of the signaling cascades involved in the initiation and progression of prostate cancer is a critical issue for the development of targeted anti-tumor therapies. Our laboratory has identified Par-4 as an interacting protein and inhibitor of the atypical PKCs, which leads to a subsequent reduction in NF-κB activity and increased cell death, consistent with the known role of the aPKCs in this pathway. Par-4 is a gene highly expressed in prostate that was initially identified in an in vitro differential screen for pro-apoptotic genes in human prostate carcinoma cell lines. We have previously shown that the genetic inactivation of Par-4 in mice leads to reduced lifespan and spontaneous tumorigenesis, suggesting that Par-4 could act as a tumor suppressor whose loss is relevant for the initiation and development of prostate cancer. In this regard, Par-4 null mice, similar to PTEN-heterozygous mice, only develop benign prostate lesions.

We now show here that concomitant Par-4 ablation and PTEN-heterozygosity lead to invasive prostate carcinoma in mice. This strong tumorigenic cooperation is anticipated in the preneoplastic prostate epithelium by an additive increase in Akt activation and a synergistic stimulation of NF-κB with an impact not only in an enhanced proliferation but also a decrease apoptotic rate in prostate epithelium. Our new data establish a novel paradigm whereby Par-4 and PTEN mutations show accelerated tumor progression through the cooperation of the loss of these tumor suppressors in prostate carcinogenesis by the activation of the Akt and NF-κB cascades. We also show that there is a concomitant loss of Par-4 and PTEN in human prostate carcinomas, and an inverse correlation with NF-κB activation in these tumor samples, suggesting the existence of a pathologically relevant biochemical and functional cooperation between these two tumor suppressors impinging the Akt and NF-κB pathways.

Poster P8-11

INFLAMMATION IN PROSTATE CARCINOGENESIS: ROLE OF THE TUMOR SUPPRESSOR PAR-4

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Prostate cancer (PCa) is one of the most common malignancies in men. Novel therapies and diagnostic techniques are needed to address this complex disease. Prostate carcinogenesis is a multistage process that includes initiation, promotion, and progression. Loss of tumor suppressor genes is a frequent initiating event that is irreversible, whereas tumor promotion and progression are susceptible to modulation, which provides a rationale for therapeutic intervention. Tumor promotion is highly regulated by the interaction between initiated cells and their microenvironment, and inflammation is a frequent and important tumor promoter. However, despite the strong evidence for an inflammatory component to the pathology of PCa, the process of inflammation and the related signaling pathways are largely unknown. Par-4 is a tumor suppressor originally identified in an in vitro differential screen of prostate cancer cells undergoing apoptosis following androgen withdrawal. Our laboratory identified Par-4 as an interacting partner of PKCzeta, which unveiled its role as a negative regulator of inflammation. The goal of this study is to explore the cooperation of the tumor suppressors Par-4 and PTEN in PCa, and the contribution of inflammation as a critical mediator of tumorigenesis. The methodology of this study is based on the use of Par-4-KO mice, developed in our laboratory, as a genetic model of increased basal inflammation. This provides an innovative and excellent tool to address the role of inflammation in PCa. Our results demonstrate that concomitant loss of Par-4 and PTEN cooperates in PCa to promote invasive carcinoma. Interestingly, the combined mutation of both tumor suppressors regulates both proliferation and survival of prostatic epithelial cells, in contrast to the cooperation between PTEN and other tumor suppressors, which only affect proliferation. This is a unique feature of Par-4 and PTEN interplay that could be explained by the synergy of the two mutations on activation of the NF-kB cascade, an important pathway in cell survival and inflammation. The inactivation of both tumor suppressors results in the synergistic stimulation of NF-kB, not only in prostatic intraepithelial neoplasia lesions but also in preneoplastic prostates. This suggests that the activation of NF-kB could be a causative mechanism in the promotion of invasive PCa. Par-4 deficiency also leads to an increase in Akt activation, and this effect is enhanced in the context of PTEN heterozygosity. Thus, the concomitant loss of PTEN and Par-4, in addition to modulating the Akt pathway, impinges on the NF-kB cascade, which could unleash signals complementary to those elicited by Akt. Two important inflammatory targets of NF-kB, the cytokine IL-6 and the chemokine IL-8, are increased in the Par-4/PTEN compound-mutant prostates. This might mediate the recruitment of inflammatory cells and facilitate an angiogenic response that could collaborate with proliferative and survival signals in the progression to an invasive phenotype. These results establish the cooperation between Par-4 and PTEN as relevant for the development of PCa and implicate the inflammatory NF-kB pathway as a critical event in this process. This work will lead to advances in our understanding of the molecular link between inflammation and prostate cancer and thus may uncover new perspectives on prostate carcinogenesis and provide novel therapeutic and preventive targets for drug discovery.

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